Functional and molecular characterization of SR45, a plant-specific splicing factor involved in sugar and stress signaling in *Arabidopsis thaliana*

Raquel Fonseca de Carvalho

Dissertation presented to obtain the Ph.D degree in Molecular Biology

Oeiras,
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RESUMO

O processo pelo qual os intrões são removidos do mARN precursor (pré-mARN) e os exões ligados entre si denomina-se *splicing*. Múltiplas formas de mARN maduro podem ser obtidas a partir de um único pré-mARN através de um mecanismo conhecido por *splicing* alternativo. Este processo permite a produção de mais do que uma proteína a partir de um único gene, contribuindo assim decisivamente para a criação de diversidade transcritómica e proteómica.

Não possuindo capacidade de locomoção, as plantas desenvolveram estratégias adaptativas únicas, quer a nível de desenvolvimento quer a nível fisiológico, que lhes permitem fazer face a um ambiente em constante mudança. O *splicing* alternativo proporciona uma forma rápida e ajustável de regular a expressão génica que pode desempenhar um papel importante nos processos de adaptação das plantas. Nos últimos anos, o número de exemplos de *splicing* alternativo descrito em plantas tem aumentado exponencialmente, havendo evidências crescentes de que este mecanismo de regulação pós-transcricional desempenha um papel fundamental na resposta das plantas ao stress ambiental.

As proteínas SR, ricas em serinas e argininas, constituem uma família altamente conservada de factores de *splicing* que desempenha um papel-chave nas etapas iniciais de montagem do spliceosoma. Estas proteínas de ligação ao ARN influenciam a escolha dos sítios de *splicing* em função da sua concentração, estando descritas em mamíferos como factores essenciais ao processo de *splicing* alternativo. No genoma de *Arabidopsis thaliana* existem pelo menos 18 genes codificando proteínas SR. O factor de *splicing* SR45, considerado até há pouco tempo uma proteína SR clássica, foi excluído desta família após a recente revisão da definição de proteínas SR.

O presente trabalho foi iniciado com o objectivo alargado de investigar o eventual papel das proteínas SR e proteínas relacionadas na resposta das plantas ao stress. Como ponto de partida, examinámos os padrões de expressão e de *splicing* dos 18 membros da família génica SR e do gene *SR45* de Arabidopsis em diferentes tecidos

e em resposta a diversas formas de stress abiótico. Os resultados obtidos mostram que a grande maioria destes genes se expressa em sementes, raízes, folhas e flores, tendo sido detectados para alguns transcritos resultantes de *splicing* alternativo. Notavelmente, tanto o frio como a aplicação exógena da fitohormona de stress ácido abscísico (ABA) afectaram a expressão dos genes SR, sendo o padrão de *splicing* de dois destes genes alterado pelo frio. Além disso, os níveis dos transcritos de SR45 foram alterados pela exposição ao ABA, salinidade elevada e stress térmico. Destes resultados concluímos que a expressão dos genes codificando tanto proteínas SR como a proteína SR45 é regulada por stress, o que indica que estes factores de *splicing* são alvos de diversas vias de transdução de sinal, podendo funcionar como coordenadores centrais da resposta das plantas a alterações ambientais. Para elucidar a relevância funcional destas proteínas de ligação ao ARN, conseguimos isolar mutantes de perda de função para quatro proteínas SR de Arabidopsis, SR34, SCL30a, SCL33 e RS31, que serão de extrema importância na futura análise funcional destas proteínas. Por outro lado, isolámos um mutante *knockout* para a proteína SR não canónica SR45, *sr45-1*, que foi já descrito anteriormente como apresentando fenótipos pleiotrópicos durante o desenvolvimento normal da planta. No entanto, a sua resposta a estímulos ambientais nunca tinha sido examinada.

Ao aprofundarmos a caracterização do mutante *sr45-1*, descobrimos que o factor de *splicing* SR45 representa um novo modulador-chave na resposta a açúcares e stress durante o desenvolvimento precoce em Arabidopsis. O *knockout sr45-1* apresenta uma paragem evidente do crescimento na presença de baixas concentrações de glucose que não afectam o genótipo selvagem, exibindo as plântulas mutantes alterações no desenvolvimento dos cotilédones e no alongamento do hipocótilo, bem como alteração na expressão de genes de resposta a açúcares. Esta hipersensibilidade à glucose é parcialmente restaurada pela presença de um inibidor da biossíntese de ABA, e de facto a mutação *sr45-1* confere maior capacidade para acumular ABA em resposta à glucose. Em concordância, a expressão de vários genes envolvidos na biossíntese e sinalização de ABA encontra-se marcadamente

sobre-induzida pelo açúcar no mutante *sr45-1*. Curiosamente, este mutante apresenta também hipersensibilidade ao ABA, à semelhança de muitos mutantes afectados na resposta aos açúcares, mas não parece apresentar problemas na percepção e sinalização do etileno. No seu conjunto, estes resultados demonstram que a proteína SR45 regula negativamente a sinalização de glucose através da repressão da via de transdução de sinal da hormona de stress ABA.

No último estudo apresentado nesta tese demonstrámos que o modo de acção da proteína SR45 na sinalização de açúcares é independente do sensor de glucose, hexocinase1 (HXK1), mas envolve regulação da degradação da proteína SnRK1.1, uma cinase envolvida na coordenação das respostas a açúcares e stress. Na verdade, e de acordo com indicações disponíveis de que plantas transgénicas sobre-exprimindo SnRK1.1 exibem também hipersensibilidade a glucose e ABA, folhas de *sr45-1* tratadas com açúcar contêm níveis significativamente mais elevados da cinase quando comparados com o genótipo selvagem. Esta elevação dos níveis de SnRK1.1 é suprimida pela presença de um inibidor do proteasoma, o que sugere um papel para a SR45 na desestabilização da proteína SnRK1.1 em resposta a açúcares. Uma descoberta-chave deste trabalho foi a de que a mutação *sr45-1* provoca alterações no *splicing* alternativo do gene *5PTase13*, que codifica uma inositol polifosfato 5-fosfatase que se sabe não só interagir com a SnRK1.1 mas também regular a sua estabilidade, apontando assim uma conexão mecanística entre a função da SR45 e a modulação dos níveis de SnRK1.1 em resposta à glucose. Por fim, tirámos partido de um painel de RT-PCR de alta resolução para detectar *splicing* alternativo e identificámos outros alvos moleculares desta proteína de ligação ao ARN.

ABSTRACT

The process by which introns are removed from the precursor mRNA (pre-mRNA) is called RNA splicing. Multiple forms of mature mRNAs can be generated from a single pre-mRNA via a mechanism termed alternative splicing, which allows more than one polypeptide product to arise from a single gene and therefore largely contributes to generating transcriptome and proteome diversity.

As sessile organisms, plants have evolved unique developmental and physiological strategies that allow them to adapt to an environment that is in constant change. Alternative splicing provides a quick and adjustable means of gene expression regulation, which is likely to be important in plant adaptive processes. The number of reported examples of alternative splicing in plants has increased exponentially over the past few years, and there is growing evidence that this posttranscriptional regulation mechanism plays a pivotal role in plant responses to environmental stress.

Serine/arginine-rich (SR) proteins constitute a highly conserved family of splicing factors that play key roles in the early steps of spliceosome assembly. These RNA-binding proteins influence splice site selection in a concentration-dependent manner and are known to be key players in mammalian alternative splicing. At least 18 genes encoding SR proteins are present in the *Arabidopsis thaliana* genome. The SR45 splicing factor, which had been regarded as a classical SR protein, now falls outside the recently revised SR protein definition.

The present thesis set out to uncover the potential role of Arabidopsis SR-related proteins in plant stress responses. As a starting point, we examined the tissue- and stress-specific expression and splicing patterns of the 18 members of the Arabidopsis SR protein gene family as well as of the non-canonical SR gene, *SR45*. The vast majority of these genes were expressed in seeds, roots, leaves and flowers, with some displaying alternatively-spliced transcripts. Importantly, both cold stress and exogenous application of the stress phytohormone abscisic acid (ABA) modulated SR

protein gene expression, and the splicing pattern of two SR genes was altered by cold. Moreover, *SR45* transcript levels were changed by exposure to ABA, salt and temperature stress. We conclude that the expression of Arabidopsis SR-related genes is stress-regulated, indicating that these splicing factors are targets of several signal transduction pathways and may function as central coordinators of plant responses to changes in the environment. To gain insight into the in vivo function(s) of these RNA-binding proteins, we were able to isolate loss-of-function mutants for four Arabidopsis SR proteins, SR34*,* SCL30a, SCL33 and RS31, which should prove invaluable in future functional analyses. In addition, we isolated a knockout mutant for the SR45 non-canonical SR protein, *sr45-1*, which has been described previously as displaying pleiotropic phenotypes during normal plant development. However, its response to environmental cues had not previously been addressed.

Our further characterization of the *sr45-1* mutant has shown that the SR45 splicing factor defines a novel key player in sugar and stress responses during early seedling development in Arabidopsis. We found that the *sr45-1* knockout exhibits a marked growth arrest in the presence of low glucose concentrations that do not affect the wild type, with mutant seedlings displaying impaired cotyledon development and hypocotyl elongation as well as altered glucose-responsive gene expression. This glucose oversensitivity is partially rescued by the presence of an ABA biosynthesis inhibitor, and indeed the *sr45-1* mutation enhances the accumulation of ABA in response to glucose. In agreement, several ABA biosynthesis and signaling genes are markedly overinduced by the sugar in the *sr45-1* mutant. Interestingly, *sr45-1* displays hypersensitivity to ABA as do many sugar-responsive mutants, but it appears to be unaffected in ethylene signaling. Taken together, these results demonstrate that SR45 negatively regulates glucose signaling by repressing the ABA stress signaling pathway.

In a final study, we showed that the mode of action of SR45 in sugar signaling is independent of a conserved glucose sensor, hexokinase1 (HXK1), but involves glucose-responsive regulation of the degradation of SnRK1.1, a protein kinase

implicated in the coordination of sugar and stress responses. In fact, consistent with a previous report that transgenic plants overexpressing SnRK1.1 also display hypersensitivity to glucose and ABA, sugar-treated *sr45-1* leaves contain significantly higher levels of the protein kinase. These enhanced SnRK1.1 levels are suppressed by the presence of a proteasome inhibitor, suggesting a role for SR45 in destabilizing the SnRK1.1 protein in response to sugars. Importantly, the *sr45-1* mutation causes changes in alternative splicing of the *5PTase13* gene, which encodes an inositol polyphosphate 5-phosphatase previously shown to interact with and regulate the stability of SnRK1.1, thus providing a mechanistic link between SR45 function and the glucose-responsive modulation of SnRK1.1 levels. Lastly, using a high-resolution RT-PCR alternative splicing panel, we pinpointed additional splicing targets of this RNA-binding protein.

LIST OF ABBREVIATIONS

ABA	abscisic acid
BBP	branch point binding protein
BiFC	bimolecular fluorescence complementation
BR	brassinosteroids
EJC	exon junction complex
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FLIP	fluorescence loss in photobleaching
FRAP	fluorescence recovery after photobleaching
GA	gibberellic acid
GFP	green fluorescent protein
HXK	hexokinase
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
mRNA	messenger RNA
NMD	nonsense mediated decay
pre-mRNA	precursor mRNA
PTC	premature stop codon
RNP	ribonucleoprotein
RNPS1	RNA-binding protein with serine-rich domain 1
RRM	RNA recognition motif
RS	arginine/serine-rich
RT-PCR	reverse-transcription polymerase chain reaction
S/RS	serine and arginine serine-rich
SA	salicylic acid
SnRK	SNF1-related kinase
snRNA	small nuclear RNA

snRNP	small nuclear ribonucleoprotein
SPXR	serine-proline-x-arginine motif
SR protein	serine/arginine-rich protein
TAIR	The Arabidopsis Information Resource
U2AF	U2 auxiliary factor
UTR	untranslated region

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CHAPTER 1

General introduction

1.1. Pre-mRNA splicing in plants

Eukaryotic genes are generally made up of coding sequences that are interrupted by stretches of non-coding sequences. The coding sequences are called exons and will give rise to the messenger RNA (mRNA), whilst the non-coding sequences are called introns. The latter are cotranscriptionally removed from the precursor mRNA (pre-mRNA) by a process called RNA splicing. The combinatorial removal of these intervening sequences via a mechanism termed alternative splicing contributes to the enlargement of the protein-coding potential in eukaryotes.

Pre-mRNA splicing is carried out by the spliceosome, a large RNA-protein complex that contains small nuclear ribonucleoproteins (snRNPs) and a large number of non-snRNP proteins. Proteomic analysis of animal spliceosomes has resulted in the identification of about 300 distinct proteins in this complex (Rappsilber et al., 2002; Zhou et al., 2002; reviewed in Jurica and Moore, 2003). Ribonucleoproteins (RNPs) are complexes of one or more proteins with a short RNA molecule that are present in all compartments of eukaryotic cells. In the nucleus there are about 200 RNPs, either residing in the nucleoplasm or in the nucleolus. snRNPs belong to a subgroup of RNPs that reside in the nucleoplasm and function primarily in splicing. Those that participate in nuclear pre-mRNA splicing contain a uridine-rich RNA, with the U snRNP being named after the type of RNA it contains. In higher eukaryotes there are two types of spliceosomes. The major U2-type spliceosome, which consists of U1, U2, U4, U5 and U6 snRNPs, catalyzes the removal of introns with canonical (GT-AG) splice sites. The minor U12-type spliceosome that contains U11, U12, U4atac and U6atac snRNPs recognizes a small percentage of introns (<1% in Arabidopsis and humans) with noncanonical splice sites.

The borders between introns and exons are marked by specific nucleotide sequences called splice sites, which delineate where splicing will occur. The 5' splice site is a specific sequence marking the exon-intron boundary at the 5' end of the intron, whereas the 3' splice site marks the intron-exon boundary at the 3' end of the intron (Figure 1.1). An additional sequence required for splicing is the branch point site, found

entirely within the intron, which is followed by a stretch of pyrimidines, the polypyrimidine tract (Figure 1.1). The most highly conserved sequences in vertebrate mRNA are the GU in the 5' splice site, the AG in the 3' splice site (canonical splice sites) and the adenosine (A) residue within the CURAY sequence (where R represents a purine and Y represents a pyrimidine) at the branch point site.
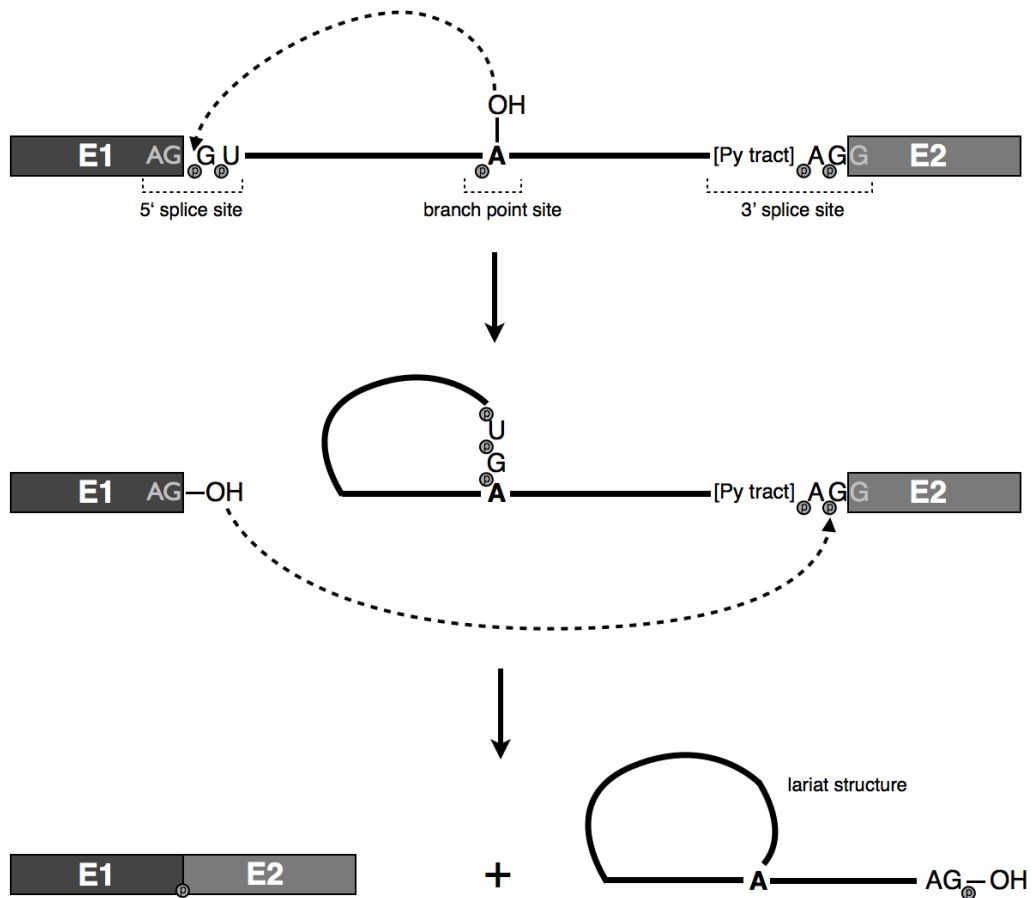
Figure 1.1. The splicing reaction.

Diagram illustrating the two steps of the splicing reaction. A pre-mRNA with a single intron represented as a line and two exons (E1 and E2) is shown on top. Two sequential transesterification reactions occur in the spliceosome as described in the text. Py tract, Polypyrimidine tract. *Adapted from Reddy (2001) and Watson et al. (2004).*

The precise excision of an intron involves two sequential transesterification reactions (Figure 1.1). The first consists of a nucleophilic attack at the 5' splice site phosphate by the hydroxyl group of the adenosine in the branch point site. This generates a bond between the first nucleotide of the intron and the adenosine in the branch point upstream of the 3' splice site to form a lariat structure and a free 3' hydroxyl group on the 5' exon. The second reaction involves another nucleophilic attack of the free 3' hydroxyl group to the phosphodiester bond at the 3' splice site, resulting in the release of the intron in a lariat form and ligation of the two exons. snRNPs recognize the 5' splice site and the branch point, bringing these sites together and catalyzing the RNA cleavage and joining reactions.

Spliceosome assembly, illustrated in Figure 1.2, proceeds in a stepwise manner and is initiated upon the binding of the U1 snRNP to the 5' splice site via base pairing between its RNA component and the pre-mRNA. The U2AF65 subunit of the U2 auxiliary factor then binds to the polypyrimidine tract, while its other subunit, U2AF35, binds to the 3' splice site. This allows U2AF65 to interact with the branch point-binding protein (BBP), thus helping it bind the branch point site. Subsequently, the U2 snRNP binds the branch point, displacing the BBP. Lastly, removal of U2AF occurs while the U4/U6*U5 tri-snRNP (U4 and U6 are held together by complementary base-pairing, while U5 is more loosely associated through protein-protein interactions) joins this complex. With the completion of spliceosome assembly, all three splice sites are in close proximity. In the next step, U6 replaces U1 at the 5' splice site and U4 is released from the complex, allowing U6 to interact with U2, forming the active site. As a result, the 5' splice site of the pre-mRNA and the branch point come in contact, facilitating the first transesterification reaction and forming the lariat structure. The second reaction is aided by the U5 snRNP, which helps bring the two exons together. In the final step, the mRNA product and the snRNPs are released, with rapid degradation of the lariat allowing recycling of the snRNPs that were bound to it. In the minor U12-type of spliceosome, the U11, U12, U4atac and U6atac snRNPs replace the U1, U2, U4 and U6 snRNPs, respectively, while the U5 snRNP is shared between the two spliceosomes.
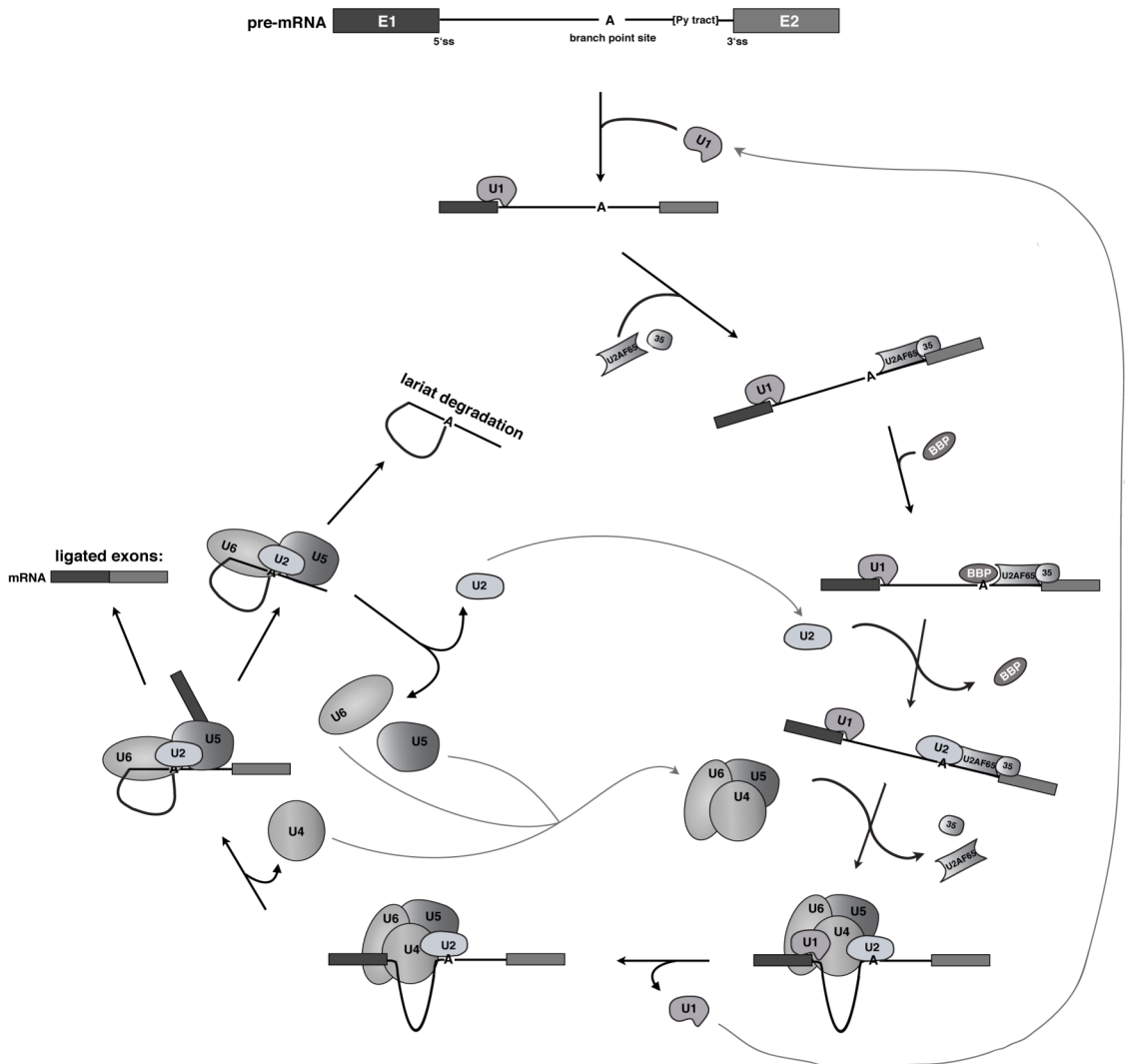
Figure 1.2. The assembly of the spliceosome.

Diagram illustrating the U2-type spliceosome cycle in mammals, probably also applicable to plants (most of the U snRNP components are highly conserved between metazoan and plant systems). The splicing of a single intron-containing pre-mRNA is shown. Sequential association of various snRNPs with a pre-mRNA results in the assembly of a spliceosome. Black arrows indicate transitions and grey arrows indicate recycling of snRNPs. 5'ss and 3'ss, 5' and 3' splice sites; Py tract, Polypyrimidine tract. *Adapted from Lorkovic et al. (2000), Reddy (2001) and Watson et al. (2004).*

1.1.1. Structural features of plant exons and introns

Plants and animals exhibit dramatic differences in their gene sizes and structures. Human genes show enormous variation in gene size (28 kb on average), have large introns (5.5 kb on average) and, like other vertebrates, relatively short exons (170 bp on average) (International Human Genome Sequencing Consortium, 2001; Sakharkar et al., 2006; Barbazuk et al., 2008). By contrast, plant genes are generally much smaller (2-4 kb on average) (Schoof et al., 2004; Haberer et al., 2005; International Rice Genome Sequencing Project, 2005; Barbazuk et al., 2008). Moreover, an intron-exon structure database, which includes analyses of the rice and Arabidopsis genomes, indicates that plant exons are slightly larger than those in humans, while their intron lengths, similar to those in yeast, Drosophila and *C. elegans*, are substantially shorter (Deutsch and Long, 1999).

Introns are abundant in plant genomes — it is estimated that about 80% of plant genes contain introns (The Arabidopsis Genome Initiative, 2000; Reddy, 2001). Furthermore, plant introns have a high UA or U content, while exons are G-rich. The UA or U enrichment of introns is important for correct recognition of splice sites and for efficient splicing of introns in plant cells (Brown and Simpson, 1998; Lorkovic et al., 2000; Reddy, 2001; Simpson et al., 2004; Reddy, 2007). Mammalian introns, which are usually not UA-rich, are generally not spliced when their processing is tested in transgenic plants or protoplasts (Barta et al., 1986; Wiebauer et al., 1988; Pautot et al., 1989). Although the majority of plant introns are rich in UA or U, introns of dicotyledonous species have a higher UA content (74% on average) when compared with monocotyledonous introns, which are longer and have an average UA content of about 59% and therefore higher GC content (Goodall and Filipowicz, 1991; Lorkovic et al., 2000; Reddy, 2001; Reddy, 2007). This results in different mechanisms of intron recognition between monocots and dicots (Goodall and Filipowicz, 1991), suggesting differences in splicing between these two lineages.

As mentioned above, four intron-defining splicing signals are important for accurate splicing of pre-mRNA in metazoans: GU in the 5' splice site, AG in the 3' splice site,

the branch point and the polypyrimidine tract. Analysis of the 5' and 3' splice sites in all introns of Arabidopsis and rice indicates that these sites are very similar to those in humans, although individual introns exhibit great variation around the highly conserved :GU and AG: dinucleotides (Brown and Simpson, 1998; Reddy, 2001; Reddy, 2007). In Arabidopsis, non-canonical splice sites occur in 0.7% of all splice sites (Alexandrov et al., 2006). The plant intron branch point site consensus is also very similar to that of vertebrates, but the typical CUR<u>A</u>Y sequence is not obvious, probably due to the variation of the position of the branch point in different introns (Brown and Simpson, 1998; Reddy, 2001; Reddy, 2007). Plants also lack the distinct polypyrimidine tract near the 3' splice site that is present in vertebrates. Instead, this region is mostly composed of uridine (Brown and Simpson, 1998; Reddy, 2001; Reddy, 2007), and these U-rich elements have been shown to function as a splicing signal or a polypyrimidine tract (Simpson et al., 2004). Thus, fundamental differences in splicing between plants and animals seem to lie at the level of intron sequence recognition early in spliceosome formation.

1.1.2. Splice site recognition: the intron and exon definition models

The short and degenerate consensus sequences at the splice and branch point sites are required for splice site recognition, but the information content in these sites alone is insufficient. Other factors such as the length and/or sequence of exons and introns are also important for the accuracy of splice site selection. Studies in animal systems indicate that other exonic and intronic regulatory sequences bound by *trans*-acting factors influence splice site selection during constitutive and alternative splicing. These regulatory sequences are divided into exonic splicing enhancers or silencers (ESEs or ESSs, respectively) and intronic splicing enhancers or silencers (ISEs or ISSs, respectively). In animals, these regulatory sequences are bound and activated by one or more of several related splicing factors such as serine/arginine-rich (SR) proteins. Much attention has been given to ESEs, which promote the inclusion (as opposed to skipping) of the exons in which they reside. In plants, little is known about

exonic regulatory sequences. Although recent computational analyses have identified ESE motifs in Arabidopsis (Pertea et al., 2007; http://www.cbcb.umd.edu/software/SeeEse/ background.html), contrary to animals no splicing event in plants has yet been shown to be enhancer-dependent.

Two different models, termed exon definition and intron definition, have been proposed to illustrate the mechanisms involved in splice site recognition (Figure 1.3) (Berget, 1995). In organisms such as humans and other vertebrates where large intron sequences separate fairly small exons, a model for exon definition has been proposed whereby interactions between factors bound at the 3' splice site and the downstream 5' splice site define the exon (Figure 1.3) (Berget, 1995; Sterner et al., 1996). According to this exon definition model, mutations in the 5' splice site should lead to skipping of a specific exon or to the activation of a proximal cryptic 5′ splice site. On the other hand, the intron definition model of splice site recognition, where factors assemble at the 5' and 3' splice sites and interact across the intron (Figure 1.3), is thought to occur in organisms with small introns in their genes (Talerico and Berget, 1994). According to this model, the sequences in the exon have little or no role in splice site recognition, and the mutation of an internal 5' splice site should lead to retention of the intron instead of exon skipping.

Because plant introns are usually short and harbor U-rich elements, their splicing is generally assumed to follow the intron definition mechanism (Lorkovic et al., 2000). However, splicing in plants could also follow the exon definition model, and this is supported by the observation of exon skipping in Arabidopsis and tomato intron mutants (Brown, 1996; Brown and Simpson, 1998; Lazarova et al., 1998). Even though there is evidence to support both these models in plants, based on the high frequency of intron retention (41% in Arabidopsis and 33% in rice, as compared with 9% in humans) (Barbazuk et al., 2008), it has been proposed that splice site recognition in plants occurs predominantly by intron definition (Reddy, 2007).
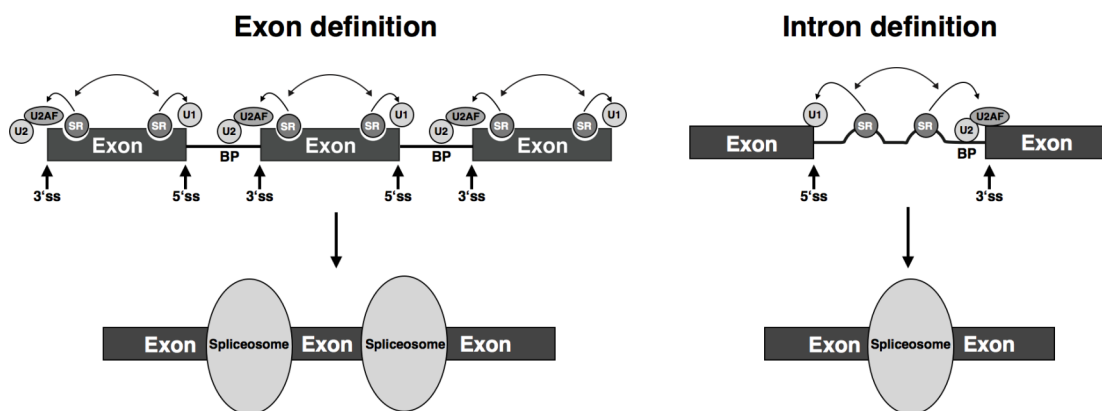
Figure 1.3. Exon and intron definition models of pre-mRNA recognition.
Diagram illustrating the two models that explain how the spliceosome accurately recognizes splice sites. In the exon definition model, the splicing machinery recognizes splice sites around an exon and assembles on the exon — splicing regulators such as SR proteins and other proteins that bind to ESEs recruit the U1 snRNP to the 5' splice site and U2AF to the 3' splice site, with the latter protein then recruiting the U2 snRNP to the branch point site. According to the intron definition model, the splicing machinery recognizes the sequence elements in the intron and assembles on the intron — splicing regulators bind to ISEs and recruit U1 snRNP and U2AF to the 5'ss and 3'ss, respectively. Half circles in exons and introns indicate ESEs and ISEs, respectively. SR, serine/arginine-rich proteins; BP, branch point site; U2AF, U2 auxiliary factor; 5'ss and 3'ss, 5' splice site and 3' splice site. *Modified from Reddy (2007).*

1.1.3. Composition of the plant spliceosome

Plant spliceosomes have never been isolated, and therefore their exact composition remains unknown (Reddy, 2007). However, most animal spliceosome components appear to be well conserved within plants. A total of 74 small nuclear RNAs (snRNAs) and 395 spliceosome-associated protein-encoding genes are predicted by sequence similarity to reside within the Arabidopsis genome (Wang and Brendel, 2004, 2006b). Most animal spliceosomal proteins in major and minor spliceosomes are also conserved in plants (Wang and Brendel, 2004; Lorkovic et al., 2005), suggesting that the composition of plant and animal spliceosomes is similar. The mechanism of splicing is also thought to be well conserved between plants and animals (Lorkovic et al., 2000; Reddy, 2001; Jurica and Moore, 2003). Nevertheless,

plant spliceosomal proteins are vastly expanded — 50% of the splicing-related genes are duplicated in Arabidopsis, and this duplication ratio of splicing-related genes increases from genes encoding snRNP proteins to genes encoding splicing regulators (Wang and Brendel, 2004). This allows for the appearance of novel proteins, indicating that the regulation of the splicing mechanism in plants (e.g. splice site recognition) may be more variable and flexible, thus enabling these sessile organisms to respond to their specific environments (Wang and Brendel, 2004; Reddy, 2007).

1.1.4. Alternative splicing in plants

Alternative splicing joins different fragments of the mRNA together to produce multiple forms of mature mRNAs from a single pre-mRNA. This strategy can give rise to more than one polypeptide product from a single gene. Among the different pre-mRNA processing possibilities, alternative splicing is the most prevalent mechanism to generate transcriptome and proteome diversity in metazoans (Graveley, 2001; Maniatis and Tasic, 2002). Some alternative splicing events are constitutive, with similar ratios of variant mRNAs in different cells, whereas others are subject to tissue-specific or developmental regulation (Lopez, 1998).

In plants, the number of reported examples of alternative splicing is increasing, although in most cases the biological significance of the different splice forms is unknown. During the past few years, the estimates of the extent of alternative splicing in the genome of flowering plants have increased from 12% to 42% (Iida et al., 2004; Campbell et al., 2006; Wang and Brendel, 2006a; Chen et al., 2007; Barbazuk et al., 2008; Filichkin et al., 2010), mostly due to an increase in the number of available Expressed Sequence Tags (ESTs) and full-length cDNA sequences, as well as to the application of high-throughput sequencing technologies to the characterization of transcriptomes.

Most alternative splicing events can be classified into five basic splicing patterns: cassette exons (also known as exon skipping), alternative 5' splice sites, alternative 3' splice sites, mutually exclusive cassette exons and retained introns (Figure 1.4). In

cassette exons, the usage of alternative 3' splice sites can lead to the inclusion or exclusion of an exon from the mRNA (Figure 1.4a). The use of alternative splice sites, either alternative proximal or distal 5' or 3' splice sites, results in an exon/intron of different length (Figures 1.4b and 1.4c). There are also cases where an entire intron is retained (Figure 1.4d). Finally, in examples of mutually exclusive cassette exons, adjacent exons can be spliced in such a way that only one of them is included at a time in the mRNA (Figure 1.4e). In humans, the most abundant alternative splicing event is cassette exons, followed by alternative 5' or 3' splice sites, while intron retention is the least common (Kim et al., 2007) (Table 1.1). By contrast, intron retention accounts for a surprising large proportion (above 30%) of the recorded alternative splicing events in Arabidopsis and rice (Zhang and Krainer, 2004; Ner-Gaon and Fluhr, 2006; Wang and Brendel, 2006a; Barbazuk et al., 2008; Filichkin et al., 2010) while cassette exons are extremely rare (Table 1.1).
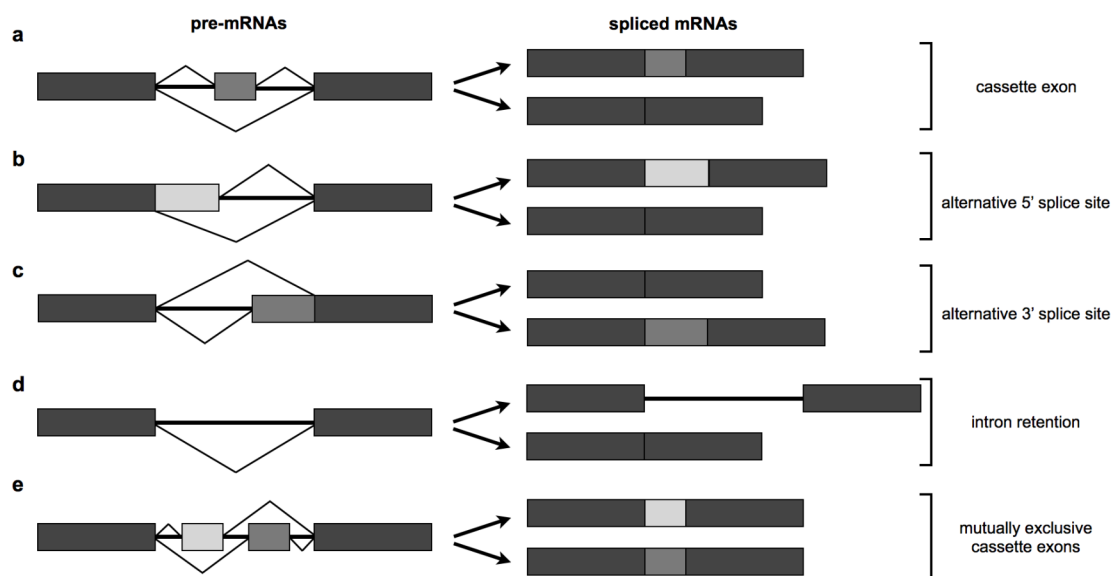
Figure 1.4. The most common types of alternative splicing.
Pre-mRNAs are shown on the left and spliced mRNAs on the right. Exons are represented by grey boxes and introns by horizontal lines. Diagonal lines indicate alternative splicing events. a) Cassette exons. b) Alternative 5' splice sites. c) Alternative 3' splice sites. d) Intron retention. e) Mutually exclusive cassette exons. *Adapted from Reddy (2007).*

Except in the case of intron retention, all the alternative splicing patterns mentioned above involve a choice between one set of splice sites competing against another. In intron retention, the competition is between splicing with intron excision or no splicing with the retention of an intron in the final mRNA. These partially spliced RNAs could therefore represent inefficient splicing and be retained in the nucleus by the binding of particular spliceosomal components. However, retroviruses have been shown to possess sequence elements that promote nuclear transport of these incompletely spliced mRNAs, and retention of a specific Drosophila intron requires binding of the Sx1 protein to both the 5' and 3' splice sites, thus allowing transport of the mRNA to the cytoplasm (Black, 2003). In addition, cases of intron retention with biological consequences have been described, as that of the P-element transcript in Drosophila (Black, 2003). Collectively, these data support the notion of a biological role for intron retention events. Importantly, in plants particular stresses and stimuli have been shown to markedly influence intron retention, and the presence or absence of the intron could either stabilize the transcript or serve to modify its biological function (Ner-Gaon et al., 2004).

Table 1.1. Frequency of common types of alternative splicing in plants and humans.

		Arabidopsis	Rice	Maize	Humans
Cassette exon		3%	11%	5%	42%
Exon extension	alt. 5' splice sites	18%	22%	22%	24%
	alt. 3' splice sites	38%	34%	38%	25%
Intron retention		41%	33%	35%	9%

Plant EST/cDNA analyses and alignments by Barbazuk *et al.* (2008); data for human alternative splicing event types reported by Kim et al. (2007). alt., alternative.

1.1.5. Alternative splicing and its role in plant stress responses

In plants, alternative splicing occurs in genes involved in a wide range of cellular processes, including transcription, splicing and signal transduction, with crucial impact on growth and development as well as in the response to biotic and abiotic stresses (Reddy, 2001; Reddy, 2007; Barbazuk et al., 2008). The effects of alternative splicing

on proteome diversity include the production of protein isoforms with loss or gain of function and/or with altered subcellular localization, stability, enzymatic activity or posttranslational modifications (Stamm et al., 2005). Furthermore, changes in alternative splicing may affect many aspects of RNA metabolism, including mRNA degradation through nonsense mediated decay (NMD), mRNA export, or mRNA recruitment to ribosomes and translation efficiency (Reddy, 2007).

As sessile organisms, plants are equipped with unique adaptive developmental and physiological strategies that allow them to respond to an environment that is in constant change. This high degree of developmental plasticity and stress tolerance is ultimately regulated by gene expression. The quickly adjustable means of gene regulation provided by alternative splicing is likely to be important in these plant adaptive processes. In fact, there is growing evidence that alternative splicing plays a pivotal role in plant stress responses. In support of such a role is the fact that alternative splicing profiles in Arabidopsis are altered under different stress conditions (Iida et al., 2004; Filichkin et al., 2010). Moreover, approximately one third of the Arabidopsis genes coding for transcripts with retained introns were found to be related to situations of stress (Ner-Gaon et al., 2004). Finally, there are numerous reports of individual genes from various species and implicated in diversified functions whose alternative pre-mRNA splicing is affected by temperature, salt, drought and/or radiation stress (reviewed in Ali and Reddy, 2008 and in Duque, 2011) (Table 1.2). More recently, heavy metal stress has been shown to change alternative splicing of members of the rice gene family of sulphate transporters (Kumar et al., 2011) (Table 1.2). Interestingly, several plant genes encoding transcription factors also undergo alternative splicing in response to stress, thereby potentially ensuring appropriate downstream stress-related gene expression (reviewed in Duque, 2011). Such is the case of the orthologs of Arabidopsis *DREB2*-type transcription factors, involved in controlling cold- and drought-responsive gene expression, which are regulated by alternative splicing under salt, drought and temperature stress in wheat, maize and rice (Table 1.2).

Table 1.2. Examples of plant pre-mRNAs that undergo stress-dependent alternative splicing changes.

Gene	Function	System	Stress	Reference
waxy	granule-bound starch synthase (GBSS)	rice (*Oryza sativa*)	heat	Larkin and Park, 1999
HSFA2	heat shock factor	*Arabidopsis thaliana*	cold	Sugio et al., 2009
invertase	enzyme that catalyzes cleavage of sucrose	potato (*Solanum tuberosum*)	cold	Bournay et al., 1996
beta-hydroxyacyl ACP dehydratase	fatty acid biosynthetic enzyme (acyl carrier protein – ACP)	black spruce (*Picea mariana*)	cold	Tai et al., 2007
CTL	low-temperature-responsive gene	rubidoux trifoliate orange (*Poncirus trifoliata*)	cold	Jia et al., 2004
e-cor (*7H8*)	early cold-regulated gene encoding a putative ribokinase	durum wheat (*Triticum durum*)	cold	Mastrangelo et al., 2005
e-cor (*6G2*)	early cold-regulated gene encoding a C3H2C3 RING-E3 ubiquitin ligase		cold and drought	
LeAOX1	alternative oxidase gene involved in the removal of stress-induced reactive oxygen species	tomato (*Lycopersicon esculentum*)	cold	Fung et al., 2006
OsIM1		rice (*Oryza sativa*)	salt	Kong et al., 2003
ZmrbohB	NADPH respiratory burst oxidase homolog (rboh) B	maize (*Zea mays*)	salt, temperature and radiation	Lin et al., 2009
OsSultr1;1; OsSultr4;1	sulphate transporters	rice (*Oryza sativa*)	heavy metal stress	Kumar et al., 2011
Wdreb2	orthologs of Arabidopsis dehydration-responsive element-binding protein 2 (DREB2)-type transcription factors	wheat (*Triticum aestivum*)	temperature, drought and salt	Egawa et al., 2006
ZmDREB2A		maize (*Zea mays*)		Qin et al., 2007
OsDREB2B		rice (*Oryza sativa*)		Matsukura et al., 2010

For the vast majority of alternatively-spliced plant genes, there is little or no information on the functional significance of the protein isoforms produced. However, functional analyses conducted with a few splice variants point to an important role of alternative splicing in photosynthesis, defense responses, flowering and also in grain quality in rice (reviewed in Reddy, 2007). A few recent studies have also shown that stress-induced changes in alternative splicing may be functionally relevant (reviewed in Duque, 2011). For example, abiotic stresses specifically induce the splice variants encoding the full-length proteins of the maize DREB2A and rice DREB2B transcription factors, which when heterologously expressed in Arabidopsis confer enhanced target gene expression and improved drought and heat-shock tolerance (Qin et al., 2007; Matsukura et al., 2010).

1.1.6. Plant SR proteins and the regulation of alternative splicing

As splice sites are short and degenerate, additional *cis*-elements located in the exon or adjacent intronic sequences are required to aid their recognition. One general class of splicing regulators that contain RNA-binding domains specific to splicing regulatory sequences such as ESEs are the members of the serine/arginine-rich SR protein family (Schaal and Maniatis, 1999). ESEs function by recruiting SR proteins, which interact with one another, the pre-mRNA, or spliceosome components to enhance recognition of adjacent splice sites. Because splicing factors bind to numerous weakly conserved sequences, a single protein can regulate multiple target genes (Stamm et al., 2005).

In animals, SR proteins have been shown to play critical roles in constitutive pre-mRNA splicing, functioning at multiple points to facilitate the splicing reaction, as well as in the regulation of alternative splicing by influencing splice site selection under different conditions in a concentration-dependent manner (Manley and Tacke, 1996; Graveley, 2000). All SR proteins have a modular domain organization and contain one or two N-terminal RNA recognition motifs (RRMs) that interact with the mRNA and a C-terminal arginine/serine-rich (RS) domain that allows protein-protein interactions

with other components of the splicing machinery. Two studies by Green and co-workers have shown that the RS domain can also directly contact with the branch point site and the 5' splice site to promote splicing (Shen and Green, 2004; Shen et al., 2004). Even though this highly conserved family of RNA-binding proteins has been identified in all metazoan species examined, it is not found in all eukaryotes (reviewed in Graveley, 2000). For example, *Saccharomyces cerevisiae* contains no SR protein, which is in accordance with alternative splicing being rare or non-existing in yeasts (Graveley, 2000; Ast, 2004). In plants, however, a multitude of SR proteins has been identified in Arabidopsis, rice and Brachypodium. Although little is known about the molecular mechanisms underlying the regulation of alternative splicing events in plants, the fact that SR proteins, which appear to be highly conserved in all genomes undergoing alternative splicing, are present in these organisms strongly suggests that they also act as key modulators of this posttranscriptional regulatory mechanism in plants. In support of such a role is the fact that a few plant SR proteins have been reported to be able to regulate splice site choices in vivo (Lazar and Goodman, 2000; Kalyna et al., 2003; Gao et al., 2004; Isshiki et al., 2006; Lopato et al., 2006; Ali et al., 2007). In general, although plant SR proteins possess highly conserved RRMs, their RS domains are more divergent, contain additional/novel domains, and seem to have evolved for more specific protein-protein interactions (Barta et al., 2010). Hence the existence of several plant-specific SR protein subfamilies, suggesting they evolved to carry out activities specific to the plant kingdom.

Based on the newly revised nomenclature of mammalian SR proteins, a new definition for plant SR proteins has been recently proposed: one or two N-terminal RRMs followed by a downstream RS domain of at least 50 amino acids and a minimum of 20% RS or SR dipeptides (Barta et al., 2010). According to this definition, Arabidopsis now contains 18 SR protein genes, while rice has 22. SR45, previously annotated as an Arabidopsis SR protein, no longer fits these criteria and has therefore been excluded from the SR protein family (Barta et al., 2010).

1.2. The Arabidopsis SR45 splicing factor

1.2.1. *SR45* gene structure

The *SR45* gene, whose structure is shown in Figure 1.5, comprises twelve exons and is reported to generate three splice variants. Unlike typical SR proteins, which contain a single RS domain at the C-terminus, SR45 includes two distinct RS domains, one on either side of the RRM (Figure 1.5). According to Zhang and Mount (2009), the N-terminal low complexity RS domain is rich in serine and arginine-serine repeats (S/RS), while the C-terminal one is rich in arginine-serine repeats (RS) and serine-proline-x-arginine motifs (SPXR) (Figure 1.5). The latter are conserved potential cyclin-dependent kinase phosphorylation sites (Songyang et al., 1994; Holmes and Solomon, 1996).

Despite its atypical domain organization, SR45 had been regarded as a classical SR protein because it is able to complement an animal in vitro splicing extract deficient in SR proteins (Ali et al., 2007). However, as this criterion was excluded from the recently proposed new mammalian SR protein nomenclature (Manley and Krainer, 2010) and due to the subsequent revision of the plant SR family classification criteria (Barta et al., 2010), SR45 is now no longer considered a member of this family of splicing regulators.

Extensive analysis of the Arabidopsis SR protein gene family by RT-PCR (Palusa et al., 2007) revealed two SR45 splice variants that differ by a 21-nucleotide sequence, which is present in splice variant 1 (*SR45.1*) but missing from splice variant 2 (*SR45.2*) due to an alternative 3' splice site at the beginning of the seventh exon (Figure 1.5). These splice variants encode very similar proteins that differ only in an eight amino acid segment. According to Zhang and Mount (2009), these eight aminoacids ($T_{218}S_{219}P_{220}Q_{221}R_{222}K_{223}T_{224}G_{225}$) are predicted to contain two potential phosphorylation sites at T_{218} and S_{219}. Recently, The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org) has annotated a third splice variant, *SR45.3*, in which the selection of an alternative 5' splice site in the ninth intron

leads to the inclusion of a 33-bp sequence, which is absent from the other two splice variants (Figure 1.5). The *SR45.3* splice variant encodes a protein with eleven additional aminoacids (YVGTHLNFFLG) that are absent from the other two protein isoforms. Both SR45 alternatively-spliced segments are in frame, neither one giving rise to a truncated form of the protein.
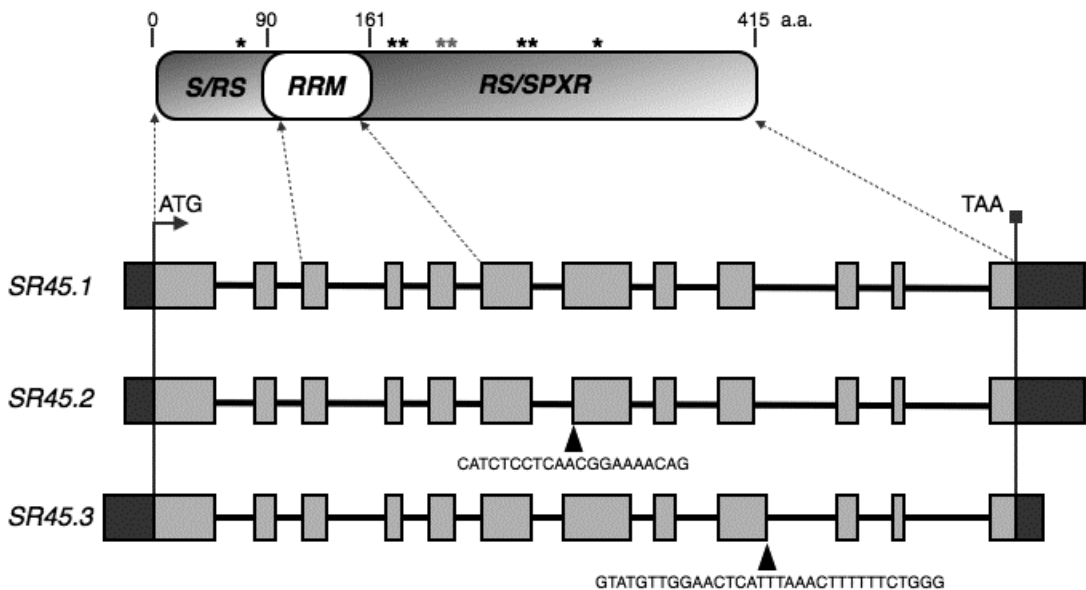
Figure 1.5. Structure of the *SR45* gene and domain organization of its encoded protein.
The *SR45* gene is reported to generate three splice variants: *SR45.1*, *SR45.2* and *SR45.3*. Exons are shown as grey boxes, with untranslated regions (UTRs) in dark grey; introns are shown as horizontal lines. The arrowheads in *SR45.2* and *SR45.3* indicate the selected alternative 3' and 5' splice sites, respectively. The 21-nucleotide sequence present in *SR45.1* and *SR45.3* but missing from *SR45.2*, as well as the 33-nucleotide sequence present in *SR45.3* but absent from the other two splice variants, are shown under each arrowhead. The schematic diagram on top shows the domain organization of the SR45 protein, with the corresponding gene positions limiting the N-terminal S/RS domain, the middle RNA recognition motif (RRM) and the C-terminal RS/SPXR domain indicated by the upward arrows. Asterisks denote the position of phosphorylation sites: in black, phosphorylation sites determined in vivo (de la Fuente van Bentem et al., 2006; de la Fuente van Bentem et al., 2008; Reiland et al., 2009); in grey, potential phosphorylation sites predicted by NetPhos 2.0 Server and by the Arabidopsis Protein Phosphorylation Site Database (Zhang and Mount, 2009). a.a., aminoacids.

Reddy and co-workers (Ali et al., 2007) identified homologs of the Arabidopsis *SR45* in rice, maize and many other flowering plants. The fact that *SR45* is exclusive to flowering plants and is absent from animals and algae suggests that this splicing factor emerged later on in evolution to perform functions that are specific to angiosperms. Interestingly, there are two *SR45* genes in rice as opposed to one in Arabidopsis, indicating that *SR45* may have undergone duplication after the divergence of monocots and dicots (Ali et al., 2007).

1.2.2. SR45 subcellular localization

Expression of SR45 as a green fluorescent protein (GFP) fusion in cultured onion and tobacco cells as well as in transgenic Arabidopsis plants has demonstrated that SR45 is localized in the nucleus, either diffusely distributed in the nucleoplasm or concentrated in speckles of variable sizes and shapes (Ali et al., 2003). In animals, it has been demonstrated that the diffused pattern of various nuclear proteins corresponds to active transcription sites and/or active sites of spliceosome assembly (Zeng et al., 1997). On the other hand, speckles, which correspond to interchromatin granule clusters and serve as storage/re-cycling sites for splicing factors (Misteli et al., 1997), deliver the splicing factors to nearby active sites of transcription (Misteli and Spector, 1998).

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) microscopy analyses have shown SR45 to be a highly mobile nuclear protein, albeit rather slower than plant canonical SR proteins, probably due to the presence and regulation of the extra N-terminal RS domain (S/RS) (Ali and Reddy, 2006). In addition, the SR45 splicing factor displays slower movement in speckles than in the nucleoplasm, suggesting that binding partners of this protein are in excess in the former. The subnuclear localization of SR45, as well as its mobility, is dependent on transcription and phosphorylation (Ali et al., 2003; Ali and Reddy, 2006). Inhibition of transcription, and therefore splicing, leads to the reorganization of SR45 into large speckles (Ali et al., 2003). Furthermore, as shown by FRAP and FLIP microscopy, the

absence of transcription reduces SR45's mobility, causing it to remain restricted to speckles instead of being recruited to active transcription sites (Ali and Reddy, 2006). This clearly illustrates a highly dynamic situation, where SR45 is recruited to sites of active splicing throughout the nucleoplasm and, when not needed during inactive transcriptional states, relocates to speckles. Importantly, stress signals are also known to control the intranuclear distribution of SR45. Upon heat shock, which is known to inhibit the transcription of various genes, SR45 is localized in enlarged nuclear speckles, whereas exposure to cold results in the disappearance of almost all speckles causing this splicing factor to be evenly distributed in the nucleoplasm (Ali et al., 2003). Both in vitro and in vivo studies have shown SR45 to be a phosphoprotein, with phosphorylation occurring at serine residues in both RS domains (Figure 1.5) (Golovkin and Reddy, 1999; de la Fuente van Bentem et al., 2006; de la Fuente van Bentem et al., 2008; Reiland et al., 2009). Upon treatment of Arabidopsis seedlings with okadaic acid (a phosphatase inhibitor) or staurosporine (an inhibitor of protein kinases), the movement of SR45 is drastically reduced, once again prompting its aggregation into large speckles (Ali et al., 2003; Ali and Reddy, 2006). The mobility of SR45 is also energy-dependent, as FRAP and FLIP analyses of ATP-depleted cells result in a dramatic reduction in the mobile fraction of SR45, both in the nucleoplasm and in speckles (Ali and Reddy, 2006).

The study of deletion mutants consisting of different domains of SR45 fused to GFP has shown that the RS domains (S/RS and RS/SPXR) of this protein, both of which contain nuclear localization signals, function independently of each other and are responsible for targeting SR45 to the nucleus, although at different strengths (Ali and Reddy, 2006). The RS/SPXR C-terminal domain alone is necessary and sufficient for speckle targeting, whereas the S/RS N-terminal domain is not involved in speckle formation. On the other hand, the RRM does not appear to carry any signals for speckle targeting, as expression of this domain alone did not induce the formation of speckles (Ali and Reddy, 2006).

1.2.3. SR45-protein interactions

SR45, first isolated as a U1-70K interacting protein in a yeast two-hybrid screen, also interacts with the SR33 SR protein and AFC2, a LAMMER-type protein kinase shown to phosphorylate SR45 and hence probably involved in the regulation of its mobility (Golovkin and Reddy, 1999). More recently, SR45 has also been shown to interact with another plant specific SR-related protein, SR45a (Tanabe et al., 2009). The SR45a protein, characterized by Tanabe et al. (2007), also possesses two RS domains separated by an RRM and interacts with U1-70K and U2AF35, which suggests that it may function in assembling spliceosomal protein components at the 5' and 3' splice sites (Tanabe et al., 2009).

The Arabidopsis U1-70K protein is one of the U1 snRNP-specific proteins and has important functions in the early steps of spliceosomal assembly by defining the 5' splice site through interactions with the U1 snRNA as well as several other U1 snRNP and non-snRNP proteins (Golovkin and Reddy, 1996, 1998, 1999; Lorkovic et al., 2004; Reddy, 2007). The in vivo interaction of SR45 with U1-70K has been shown by co-localization studies and bimolecular fluorescence complementation (BiFC) assays (Ali et al., 2008). Moreover, domain deletion mutants coupled with BiFC analyses suggest that when SR45 is bound to RNA it requires both RS domains for the interaction with U1-70K (Ali et al., 2008). Thus, and because each RS domain interacts independently with U1-70K, it is likely that the S/RS and RS/SPXR domains interact differentially with U1-70K at distinct stages of splicing, which may be important for spliceosome rearrangement during the splicing reaction (Ali et al., 2008). Alternatively, as SR45 is also known to interact with SR33, it is possible that one domain binds to U1-70K while the other binds to another SR protein during assembly of the spliceosome (Ali et al., 2008).

Finally, using a combination of FRAP and BiFC, Ali et al. (2008) have shown SR45/U1-70K complexes to exhibit very slow mobility. These observations are in contrast to the individual mobilities of U1-70K (Ali et al., 2008) or SR45 (Ali and Reddy, 2006), indicating that the complex resides in speckles for a longer time than U1-70K or

SR45 alone, probably in a storage form. This is consistent with the proposed role of speckles as storage sites for pre-mRNA processing factors. The subnuclear distribution of the SR45/U1-70K complex is regulated by protein phosphorylation, but the interaction is not disrupted by phosphorylation inhibition (Ali et al., 2008).

1.2.4. Involvement of SR45 in plant development

In 2007, Ali et al. reported the isolation and characterization of the first loss-of-function mutant for SR45, *sr45-1*, showing that this splicing factor plays an important role during normal plant development. Indeed, the authors found that depletion of *SR45* levels causes a reduction of plant size and a general delay in development — slower root growth and late flowering — as well as morphological defects, such as elongated and curly leaves at the seedling stage and altered petal and stamen numbers in adult plants.

Regarding the late flowering phenotype, physiological, molecular and genetic analyses have led Ali et al. (2007) to place the SR45 splicing factor in the autonomous flowering pathway. Firstly, the late flowering phenotype of *sr45-1* was observed under long and short day photoperiod, and prolonged cold treatment of the seeds rescued this phenotype. This is in agreement with other described autonomous flowering pathway mutants, which also flower late under both long and short day conditions and are rescued by vernalization. Secondly, *sr45-1* showed an elevated level of the *FLOWERING LOCUS C* (*FLC*), just like all other reported autonomous pathway mutants. Lastly, expression of *CONSTANS* (*CO*), a gene belonging to the photoperiod pathway, was unchanged in the *sr45-1*, indicating that SR45 is not operating in this pathway. As for the leaf morphology of *sr45-1*, Ali et al. (2007) observed that it resembles that of mutants that are defective in leaf expansion. However, microarray data and RT-PCR analyses of several known genes involved in controlling leaf morphology did not reveal significant SR45-dependent changes in their expression levels. On the other hand, comparison of cell shape and number in leaves of *sr45-1* and wild-type plants indicated that SR45 likely affects both cell expansion and division.

Indeed, microarray analyses revealed that genes involved in diverse cellular processes known to affect cell expansion are moderately altered in the mutant background. Although the number of stomata was increased in *sr45-1,* the genes regulating stomatal density on which SR45 might act remain unknown. Similarly, the alteration of organ numbers in Arabidopsis flowers has been linked to lesions in several genes but none of these genes were differentially expressed between *sr45-1* and the wild type, suggesting that other loci are affecting flower organ numbers in an SR45-dependent manner. Gene expression analyses by microarray experiments do not allow the detection of alternatively-spliced transcripts. Nevertheless, while confirming microarray data on genes differentially expressed in *sr45-1* by RT-PCR, Ali et al. (2007) also checked the alternative splicing patterns of the flowering related-genes *FCA* and *FLC*. The *FCA* gene is known to regulate flowering by producing multiple transcripts (Macknight et al., 2002), but no differences in its splicing pattern were found between *sr45-1* and wild-type plants. Similarly, the alternative splicing of *FLC*, for which there are at least four annotated splice variants, was unchanged in the *sr45-1* mutant. Finally, because splicing-related genes are themselves known to be targets of alternative splicing regulation (Lopato et al., 1999; Lazar and Goodman, 2000; Kalyna et al., 2003; Palusa et al., 2007), Reddy and co-workers (2007) analyzed the splicing profiles of the 18 Arabidopsis SR genes in the *sr45-1* background. Indeed, the *sr45-1* mutation was found to affect alternative splicing of five SR protein genes (*SR30, RS31, RS31a, SR34* and *SR34b*) indicating that SR45 directly or indirectly targets these SR pre-mRNAs. For most cases, these alternative splicing differences were due to a reduction in the usage of the distal 3' splice sites that generate a smaller transcript and an increase in the usage of the proximal 3' splice sites in the *sr45-1* mutant. This suggests that the SR45 protein favors the usage of the distal 3' alternative sites and is thus responsible for a specific balance of alternative transcripts. Ali et al. (2007) proposed that the splice variants encoded by SR protein genes modulate the splicing and/or other RNA processing activities of genes involved in the developmental processes affected in the *sr45-1* mutant.

1.2.5. Biological significance of *SR45.1* and *SR25.2*

In a recent study, Zhang and Mount (2009) showed that two alternatively-spliced isoforms of SR45, SR45.1 and SR45.2, perform distinct biological functions during plant development, hence ascribing functional significance to alternative splicing of this Arabidopsis gene. In vivo studies using stable transgenic lines expressing *SR45.1-GFP* and *SR45.2-GFP* constructs in the *sr45-1* background revealed that isoform 1 rescues solely the narrow flower petal phenotype whereas isoform 2 exclusively restores the delay in root growth. Thus, although SR45.1 and SR45.2 are being expressed in all tissues and similarly throughout the nucleoplasm (Zhang and Mount, 2009), they complement *sr45-1* defects only in specific tissues (root or inflorescences). The fact that the alternatively-spliced segment in SR45.1 possesses two putative phosphorylation sites could explain the functional differences between the two isoforms. Indeed, Zhang and Mount (2009) observed that when both phosphorylation sites were mutated (T_{218} and S_{219}), the root phenotype was rescued instead of the flower phenotype. On the other hand, mutation of only S_{219} still rescued the flower phenotype, showing that T_{218} alone is important in distinguishing SR45.1 function and that phosphorylation plays an important role in the function of the arginine/serine-rich SR45 protein.

1.2.6. SR45 as an RNPS1 homolog

In animals, the exon junction complex (EJC) is a conserved multiprotein complex that assembles on spliced mRNAs upstream of exon-exon junctions and can regulate their subsequent export, translation, localization or degradation (Tange et al., 2004). A proteomic analysis of Arabidopsis nucleoli has identified six components known from animal studies to be part of the EJC: Aly2, UAP56-2, RNPS1, Y14, Mago and eIF4A-III (Pendle et al., 2005). In this study, among the putative Arabidopsis orthologs for these EJC components, SR45 was listed as the counterpart of RNPS1. Based on its domain structure and on blast searches of its RRM, Zhang and Mount (2009) have also proposed that SR45 is an ortholog of RNPS1 in Arabidopsis.

The RNA-binding protein with serine-rich domain, RNPS1, purified from HeLa cells is a nuclear splicing activator protein (Mayeda et al., 1999) and indeed a component of the EJC (Le Hir et al., 2000). It has also been found to be a nucleocytoplasmic shuttling protein involved in NMD and mRNA transport (Lykke-Andersen et al., 2001), as well as to prevent genomic instability by working with the SFRS1 (ASF/SF2) SR protein to form RNP complexes on nascent transcripts (Li et al., 2007a).

Owing to its ability to promote a variety of alternative splicing events in a substrate-specific manner, RNPS1 appears to be a versatile splicing regulator for a wide variety of alternatively-spliced human genes (Sakashita et al., 2004). In accordance, it interacts with four factors that are known to be directly or indirectly involved in animal pre-mRNA splicing: SFRS11 (p54), an SR protein; hTra2, a purine-rich ESE-binding factor; hLuc7, a human homolog of a yeast U1 snRNP component; and pinin, which localizes in nuclear speckles and might associate with U2 snRNP proteins (Sakashita et al., 2004).

In 2007, Ali et al. checked the splicing ability of SR45 in vitro by expressing it as a His-tag fusion in insect cells and analyzing its splicing activity in S100 HeLa cell cytosol extracts with a β-globin pre-mRNA substrate. The purified SR45 protein was able to activate splicing of the β-globin pre-mRNA in a concentration-dependent manner and at a comparable level to recombinant human SRSF1 (ASF/SF2), thus demonstrating SR45 to function as an essential splicing factor. Nevertheless, given the close homology with RNPS1, SR45 functions could extend beyond the regulation of splicing. As a splicing factor, this RNA-binding protein can bind to a target mRNA, recruit spliceosomal components and thus modulate alternative splicing of a gene. However, even though nucleocytoplasmic shuttling for this protein has never been reported, it cannot be excluded that SR45 is also involved in other aspects of RNA metabolism or in the maintenance of genomic stability.

1.3. Sugar sensing and signaling in plants

Sugars, the prime carbon and energy sources for most cell types, play a central role in plant life. These essential components of plant nutrition are produced by photosynthesis — the photosynthate — in the chloroplasts of source tissues and then exported to the cytosol where they can be used in glycolysis for respiration to meet the energy needs of the cell (catabolism) or to provide carbon skeletons for the synthesis of other compounds required by the cell (anabolism). They can also be converted into sucrose that is transported to sink tissues or converted into storage compounds such as starch. Besides this essential role in photosynthetic activity and plant metabolism, sugars also function as central signaling molecules, influencing gene expression, metabolism, stress responses, and plant growth and development.

1.3.1. Sugar signals and responses in plants

Photosynthesis occurs mainly in leaf mesophyll cells (source tissues) and photosynthate is transported to meristems and developing organs such as growing young leaves, roots, flowers, fruits and seeds (sink tissues). The modulation of gene expression and enzyme activities in both source and sink tissues is rigorously coordinated by sugar levels — low sugar status enhances photosynthesis as well as reserve mobilization and export, whereas the abundant presence of sugars promotes growth and carbohydrate storage (Koch, 1996). For instance, sugar signals are crucial in starch synthesis and breakdown — excess photosynthate during the day is transiently stored as starch as a result of the sugar-dependent activation of ADP-glucose pyropohsphorylase (AGPase), while during the night reduced leaf sucrose content will elicit starch breakdown (reviewed in Rolland et al., 2006).

Sugars can signal alterations in gene expression in a similar way as hormones. However, while hormones are functional in the nano- to micromolar range, sugars are present in the milimolar range. A wide variety of genes are sugar-regulated at the transcriptional level, including genes involved in photosynthesis, carbon and nitrogen metabolism, stress responses and secondary metabolism in different plant species.

Regarding plant growth and development, sugars have important signaling functions throughout all stages of the plant's life cycle, including embryogenesis, seed germination, seedling development, hypocotyl elongation, leaf formation, nodule growth, pollen development, tuber formation and adventitious root formation, juvenile-to-adult phase transition, flowering and induction of senescence (reviewed in Gibson, 2005).

Although sucrose is the major photosynthetic product and transport sugar in plants, most sugar effects on growth and metabolism can be attributed to the action of its hydrolytic hexose products, glucose and fructose, as well as their downstream metabolic intermediates. Nevertheless, sucrose and trehalose have also been shown to regulate responses affecting plant growth, development and stress resistance (reviewed in Rolland et al., 2002; Rolland et al., 2006; Ramon et al., 2008).

1.3.2. Sugar sensing

To activate signal transduction pathways sugars first have to be sensed. Several sensors that monitor internal and extracellular sugar levels have been proposed in plants (Figure 1.6).

Hexokinase (HXK), being the first enzyme in glycolysis, is involved in the breakdown of carbohydrates to fuel respiration and provide carbon intermediates to numerous anabolic pathways, catalyzing the following reaction: hexose + ATP $\rightarrow$ hexose-6-phosphate + ADP. In addition, studies on photosynthetic gene expression using various sugars and the HXK-specific competitive inhibitor mannoheptulose, as well as the characterization of *HXK* Arabidopsis sense and antisense and tomato overexpression transgenic lines (Jang and Sheen, 1994; Jang et al., 1997; Dai et al., 1999; Pego et al., 1999), have supported the involvement of plant HXK in sugar sensing and signaling (Figure 1.6). The isolation of the *glucose insensitive2* (*gin2*) mutant, which maps to the Arabidopsis *HKX1* gene (Moore et al., 2003), also provided compelling evidence for HXK as a sugar sensor. HXK is therefore proposed to be a dual-function enzyme with both catalytic and regulatory functions. In fact, by using

Arabidopsis transgenic lines with altered levels of HXK, Xiao et al. (2000) demonstrated that gene expression can be correlated with either a HXK1-mediated signaling-dependent function or a HXK1-mediated metabolic-dependent function. The authors showed that glucose repression of photosynthetic genes such as *CAB1* (chlorophyll a/b binding protein)*, PC* (plastocyanin) or *RBCS* (rubisco) is dependent on the signaling function of HXK1, as expression of these genes is further reduced in *35S-AtHXK1* overexpression lines, whereas in plants overexpressing yeast *HXK2* (*YHXK2*) or in *35S-antiAtHXK1* lines, little to no repression occurs. On the other hand, glucose induction of *PR* (pathogen-related) genes depends on the levels of an unknown metabolite downstream of HXK in the glycolytic pathway. Loss of *PR* gene induction in *35S-antiAtHXK1* lines indicated the requirement of HXK1. However, as the induction of these genes was exaggerated to the same extent in both *35S-YHXK2* and *35S-AtHXK1* overexpression lines, Xiao *et al.* (2000) suggested the glucose induction of these genes to depend on HXK catalytic activity but not on the signaling function of HXK1. Furthermore, two catalytically inactive HXK1 alleles are able to mediate the developmental arrest of Arabidopsis seedlings on high glucose concentrations as well as glucose repression of chlorophyll accumulation and photosynthetic gene expression in the *gin2* mutant background, which unambiguously shows not only that HXK1 is a true sensor in plants but also that glucose signaling can be uncoupled from glucose metabolism (Moore et al., 2003).

Among plant *HXK* families, that of rice comprises at least nine expressed genes (Cho et al., 2006a), tomato at least four (Kandel-Kfir et al., 2006) and Arabidopsis likely six (Rolland et al., 2002; Claeyssen and Rivoal, 2007). Three of the six members of the Arabidopsis *HXK* gene family encode hexokinase-like (HKL) proteins and lack catalytic activity (Karve et al., 2008). HXK proteins are reported to occur in the cytosol, mitochondria, plastids, nuclei and Golgi (Figure 1.6) (reviewed in Rolland et al., 2002; and Ramon et al., 2008).

In Arabidopsis, HXK1 is predominantly associated with the mitochondria and has been proposed to function in actin filament reorganization and thereby influence the formation and/or stabilization of cytoskeleton-bound polysomes (Balasubramanian et

al., 2007; Balasubramanian et al., 2008). However, a small proportion of the HXK1 protein has also been reported to localize to the nucleus, where it appears to control the expression of specific photosynthetic genes as a transcriptional corepressor by creating a nuclear complex core with the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B) (Figure 1.6) (Cho et al., 2006b). Together, these data suggest that the sensor protein may translocate between mitochondria and the nucleus, modulating gene and/or protein expression from both locations. In rice, OsHXK5 and OsHXK6 have been recently shown to also act as glucose sensors and to have a predominantly mitochondrial association but possible nuclear function (Cho et al., 2009a; Cho et al., 2009b). However, in contrast to AtHXK1, both OsHXK5 and OsHXK6 contain a predicted nuclear localization signal.

An HXK1-independent pathway for glucose signal transduction has additionally been described based on the observation that the effect of glucose on the expression of genes encoding ADP-glucose pyropohsphorylase (AGPase), chalcone synthase (CHS), phenylalanine ammonia-lyase (PAL) and asparagine synthase (ASN) is similar in both the wild type and Arabidopsis *35S-HXK1*, *35S-antiHXK1* and *35S-YHXK2* transgenic lines, indicating that the sugar effect is independent of both HXK1 signaling and metabolic functions (Xiao et al., 2000). Furthermore, glucose inhibition of seed germination has been shown to operate independently of HXK function (Dekkers et al., 2004).

A number of studies have also provided strong evidence for an HXK-independent, sucrose-specific signaling pathway. In sucrose-specific pathways, the effect of sucrose cannot or can only partially be mimicked by the sucrose breakdown products glucose and fructose or by other sugars. A few examples are the sucrose-induced expression of a putative chloroplast glucose-6-phosphate/phosphate translocator, or the sucrose-specific repression of a gene that encodes a sugar beet proton-sucrose symporter (reviewed in Ramon et al., 2008). In addition, sucrose has been suggested to regulate the translation of the ATB2 protein encoded by a group S basic region leucine zipper transcription factor, bZIP11 (Wiese et al., 2004). Interestingly, nonmetabolizable sucrose analogs such as palatinose and turanose can also affect carbohydrate

metabolism and gene expression, suggesting the existence of a disaccharide sensing system at the plasma membrane (Figure 1.6) (reviewed in Rolland et al., 2002 and Ramon et al., 2008). Although the nature of a sensor for sucrose is still obscure, an atypical SUT2/SUC3 was proposed to act as a sucrose sensor in analogy to the SNF3 and RGT2 glucose sensors in yeast (Figure 1.6) (Barker et al., 2000).
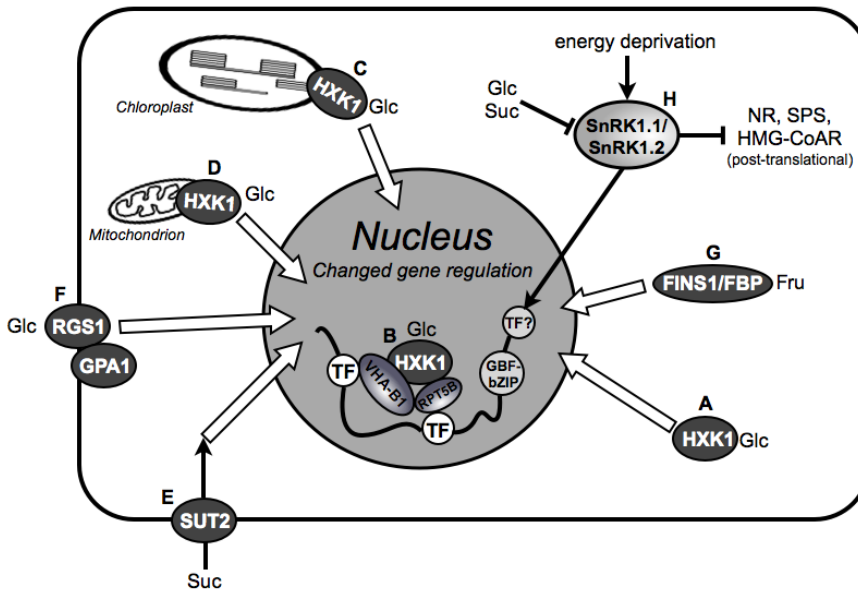
Figure 1.6. Sugar and energy sensing in Arabidopsis — proposed sensors/receptors.
A. The HXK1 protein has been implicated in glucose signaling activity in the cytosol. **B.** HXK1 is also present in the nucleus, where it forms complexes with unconventional partners (VHA-B1 and RPT5B) and transcription factors, controlling transcription. **C.** and **D.** The HXK1 protein also has signaling functions in connection with organelles. **E.** Sucrose, the main transported sugar in Arabidopsis, is found to have specific effects not triggered by its hydrolytic products, glucose and fructose; the SUT2/SUC3 sucrose transporter has been proposed to act as a sucrose sensor. **F.** G-protein coupled receptor signaling by RGS1 and GPA1. **G.** The FINS1/FBP protein has been implicated in fructose signaling in Arabidopsis. **H.** SnRK1.1/SnRK1.2 (AKIN10/AKIN11) play a key role in plant energy signaling, mediating reprogramming of transcription (in part through bZIP transcription factors) and controlling enzymes post-translationally; sugars (glucose and sucrose) have a repressive effect on SnRK1.1/SnRK1.2.
HXK, Hexokinase; FINS1/FBP, Fructose Insensitive 1/Fructose-1,6-Bisphosphatase; NR, nitrate reductase; SPS, sucrose phosphate synthase; HMG-CoAR, 3-hydroxyl-3-methylglutaryl-coenzyme A reductase; TF, transcription factor; Glc, glucose; Fru, fructose; Suc, sucrose. *Adapted from Ramon et al. (2008) and Hanson and Smeekens (2009).*

A few lines of evidence have pointed to the existence of a G-protein-coupled receptor as a glucose sensor in plants. In yeast and mammals, extracellular sucrose and glucose can be perceived by G-protein-coupled receptors (GPCRs) linked to heterotrimeric guanine nucleotide-binding proteins (G-proteins) (Lemaire et al., 2004; Chandrashekar et al., 2006). Arabidopsis mutants in the G-protein-interacting membrane protein RGS1 gene were shown to be impaired in glucose sensing, hence suggesting that RGS1 may also function as glucose sensor (Figure 1.6) (Grigston et al., 2008). In accordance, mutants in the G-protein α-subunit gene *GPA1* are hypersensitive to the glucose inhibition of germination (Huang et al., 2006), and glucose has been reported to alter the interaction of GPA1 and RGS1 in vivo (Johnston et al., 2007).

Fructose signaling in plants has remained largely unexplored, but a very recent study, taking advantage of a cell-based functional screen using transient expression of the *Arabidopsis thaliana* mesophyll protoplast system, identified FINS1 (Fructose Insensitive1), a putative Fructose-1,6-Bisphosphatase (FBP), to play a crucial role in fructose signaling responses (Figure 1.6) (Cho and Yoo, 2011). These authors have shown that fructose also modulates early seedling development, but that fructose and glucose signaling rely on distinct sensors, as the glucose-insensitive *gin2* mutant exhibits normal fructose sensitivity.

1.3.3. Sugar signal transduction

Sugars, upon being detected by sensors located at the plasma membrane or at a specific intracellular location, generate a signal that is transduced, ultimately resulting in appropriate responses that include changes in gene expression and altered enzyme activities. Although the components involved in sugar signaling pathways are still not well characterized in plants, biochemical, physiological, and genetic approaches have revealed that plant sugar signaling networks directly or indirectly interact with hormone signaling pathways and several intermediates such as Ca^{2+}. Protein phosphatases and kinases are also known to contribute to sugar signal transduction.

1.3.3.1. Genetic dissection of sugar signaling

A genetic approach, using Arabidopsis as a model system, offers distinct strategies to dissect the complex mechanisms that underlie sugar signaling in plants. Based on reporter gene expression or developmental phenotypes during germination and seedling development, a large collection of sugar signaling mutants has been isolated in Arabidopsis (Smeekens, 2000; Rolland et al., 2002; Leon and Sheen, 2003; Gibson, 2005; Ramon et al., 2008). However, because these screens are all performed with seedlings, the information on signaling networks is necessarily limited to the early developmental stages.

In reporter-based screening tools, promoters of sugar-induced or sugar-repressed genes are linked to reporter genes, allowing the selection of sugar unresponsive or hyperresponsive mutants. For example, the *sucrose uncoupled* (*sun*) mutants show high activity of a sucrose-repressed plastocyanin (*PC*) promoter fused to a luciferase reporter in the presence of 3% sucrose (Dijkwel et al., 1997). Similarly, the *reduced sugar response* (*rsr*) (Martin et al., 1997) and the *impaired sucrose induction* (*isi*) (Rook et al., 2001) mutants were selected based on the loss of their ability to induce patatin *Pat(B33)* or AGP large subunit *ApL3* gene expression, respectively, on sucrose-containing medium.

The observation that Arabidopsis seedling development is arrested at high glucose or sucrose concentrations also allows for an effective mutant isolation strategy. Examples of this type of screening are the *glucose insensitive* (*gin*) mutants that develop normally in the presence of 6% glucose (Van Oosten et al., 1997). However, the use of high concentrations of sugar has raised concerns about physiological relevance, significance, and specificity (Leon and Sheen, 2003; Rook and Bevan, 2003). The high osmolarity caused by high concentrations of glucose together with the nitrate present in Murashige and Skoog (MS) growth medium appear to complicate the sugar responses (Moore et al., 2003; Cho et al., 2006b). When the nitrate effect can be minimized, the sugar effect is observed at more physiological concentrations — for HXK1-mediated growth promotion in seedlings, 0.2% glucose in 0.1xMS medium is

sufficient to provide the glucose signal requirement in vivo (Moore et al., 2003; Cho et al., 2006b). In addition, to evaluate glucose responses that are more physiologically relevant, Cho et al. (2010) have confirmed that in 2% glucose without MS medium wild-type seedlings exhibit the same developmental arrest as in normal MS medium supplemented with 6% glucose, whereas the *HXK1* null mutant *gin2* still shows glucose insensitivity.

Other signaling mutants have been identified in unrelated genetic screens (reviewed in Ramon et al., 2008). For example, the *hypersenescence1* (*hys1*) mutant was isolated in an early senescence screen and displays a sugar hypersensitive phenotype (Yoshida et al., 2002). Another sugar mutant, *pleiotropic regulatory locus1* (*prl1*) displays complex hormone and sugar response phenotypes as well as severe defects in plant defense (Nemeth et al., 1998; Palma et al., 2007). Finally, dark-grown *high sugar response8* (*hsr8*) mutant seedlings show glucose-hypersensitive hypocotyl elongation and development (Li et al., 2007b). Interestingly, this phenotype is suppressed in the *prl1* background, suggesting that PRL1 acts as a positive regulator for the specific glucose effects observed in etiolated *hsr8* seedlings. The fact that *HSR8* is allelic to *MUR4*, which encodes an enzyme involved in arabinose synthesis, indicates that failure in the synthesis of this five-carbon sugar and consequent changes in cell wall composition could have a strong impact on sugar signaling (Li et al., 2007b).

Despite different designs for genetic screens, many independently isolated sugar-insensitive mutants are allelic, suggesting the use of conserved mechanisms in plant sugar responses (Rolland et al., 2002).

1.3.3.2. Sugars and phytohormones

Genetic and molecular studies of sugar signaling mutants in Arabidopsis have uncovered many unexpected links between sugar and plant hormone signaling (Rolland et al., 2002; Leon and Sheen, 2003; Ramon et al., 2008). ABA mediates a post-germinative developmental arrest checkpoint that enables the germinated

embryos to cope with new, adverse growth conditions (Lopez-Molina et al., 2001). As mentioned above, during Arabidopsis early seedling development high levels of exogenous sugars similarly repress hypocotyl elongation, cotyledon greening and expansion, and shoot development. Moreover, a large number of mutants with abnormal sugar responses are also affected in ABA signaling or production, and several glucose-insensitive mutants have turned out to be allelic to ABA mutants (Table 1.3).

Table 1.3. Allelism between sugar and hormone signaling mutants in Arabidopsis.

Mutant	Allelic to	Phenotype	Protein	Function	Reference
gin1	*aba2/isi4/sis4*	Glc insensitive, growth retardation, wilty, seed defect	SDR1	ABA biosynthesis	Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002
gin2		Glc insensitive, growth retardation, delayed leaf senescence, seed defect	HXK1	Glc sensor; Hexose phosphorylation	Moore et al., 2003
gin4	*ctr1/sis1*	Glc insensitive, growth retardation, constitutive triple response	CTR1	Raf-like MAPKKK, negative regulator of ethylene signaling	Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002
gin5	*aba3/isi2*	Glc insensitive, wilty	MCSU	biosynthesis of Mo-cofactor	Arenas-Huertero et al., 2000; Rook et al., 2001
gin6	*abi4/isi3/sis5/sun6*	Glc insensitive, ABA insensitive, osmotolerant, salt resistant	AP2-like TF	ABA signaling	Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001

Abbreviations: Glc, glucose; ABA, abscisic acid; HXK, hexokinase; CTR, constitutive triple response, Mo, molybdenum; MCSU, molybdenum-cofactor sulfurase; SDR, short-chain dehydrogenase/reductase. Mutant names: *gin*, glucose insensitive; *aba*, ABA deficient; *abi*, ABA insensitive; *isi*, impaired sugar induction; *sis*, sugar insensitive; *sun*, sucrose uncoupled. *Adapted from Léon and Sheen (2003) and Ramon et al. (2008).*

Although the ABA biosynthesis mutant *aba1* displays a strong *gin* phenotype (Arenas-Huertero et al., 2000), no alleles in sugar responses have yet been reported for this gene. However, the *gin1/isi4/sis4* and *gin5/isi2* glucose-insensitive mutants (Table 1.3) are allelic to two other ABA biosynthesis mutants (*aba2* and *aba3*),

displaying lower endogenous ABA levels and reduced seed dormancy (Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001). When overexpressed in the *gin1* or *gin5* backgrounds, HXK1 fails to alter the glucose insensitivity of these mutants, indicating that ABA acts downstream of the HXK1 glucose sensor (Zhou et al., 1998; Arenas-Huertero et al., 2000). The fact that ABA accumulation and the transcript levels of several ABA biosynthesis genes are significantly increased by glucose (Cheng et al., 2002) suggests that glucose-specific accumulation of ABA is required for glucose signaling during early seedling development. This is mediated by a synergistic effect of glucose and ABA and an ABA positive feedback control loop – a number of ABA biosynthesis genes (*ABA1, ABA2, ABA3, NCED3,* and *AAO3*) require endogenous ABA for their glucose activation and, except for *ABA2*, are all activated by ABA (Cheng et al., 2002).

Several mutants initially identified as showing sugar response defects are also affected in ABA signaling. For instance, the *gin6* mutant was found to contain a T-DNA insertion in the promoter of *ABI4* (*ABA INSENSITIVE 4*) and shows decreased sensitivity to ABA inhibition of germination and altered seed-specific gene expression (Arenas-Huertero et al., 2000). Furthermore, *sun6, sis5* and *isi3* isolated in screens for glucose-insensitive mutants, were found to be allelic to *ABI4* (Table 1.3) (Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). Three other ABA signaling components, *ABI3*, *ABI5* and *ABI8* are also involved in sugar response. Mutations in the *ABI3* and *ABI5* transcription factors lead to glucose insensitivity (Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000; Laby et al., 2000; Finkelstein et al., 2002), whereas overexpression of either gene results in glucose hypersensitivity (Brocard et al., 2002; Yuan and Wysocka-Diller, 2006). In addition, Huang et al. (2008) have reported the *sis10* sugar response mutant to be allelic to *ABI3.* Finally, a mutation in *ABI8*, encoding a small protein with unknown function, confers a phenotype that is partially rescued by glucose (Brocard-Gifford et al., 2004).

Despite evident crosstalk between sugar and ABA signaling, the fact that not all ABA signaling mutants are glucose responsive during early seedling development indicates that there are multiple pathways for glucose and ABA signaling (Arenas-

Huertero et al., 2000). Moreover, the inhibition of germination by ABA is reduced by low sugar concentrations (Finkelstein and Lynch, 2000). Thus, ABA and sugar signaling networks may be linked differently depending on cell types, developmental stages, physiological state, and environmental cues (Rolland et al., 2002).

An interaction between sugars and the phytohormone ethylene was first suggested by the observation that the *gin* phenotype could be mimicked by treatment of wild-type seedlings grown in the presence of 6% glucose with the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) (Zhou et al., 1998; Gibson et al., 2001). Other lines of evidence, such as allelism between ethylene and glucose responsive mutants, further support such an interaction. For example, the *constitutive triple response 1* (*ctr1*) mutant is allelic to *gin4/sis1* (Table 1.3), and the *ethylene overproduction 1* (*eto1*) mutant is insensitive to the glucose repression of early seedling development, whereas the ethylene insensitive mutants *etr1, ein2, ein3* and *ein6* exhibit glucose hypersensitivity (Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002; Yanagisawa et al., 2003). In addition, the *etr1 aba2* and *ein2 aba2* double mutants display glucose insensitivity, an indication that ethylene also affects glucose signaling partially through ABA to promote germination and seedling development (Zhou et al., 1998; Ghassemian et al., 2000; Cheng et al., 2002). Importantly, Yanagisawa *et al.* (2003) have shown the stability of a key regulator of ethylene signaling, the EIN3 (ETHYLENE INSENSITIVE 3) transcription factor, to be differentially regulated by glucose and ethylene. Using both maize and Arabidopsis leaf mesophyll protoplasts as well as transgenic Arabidopsis seedlings, these authors found that glucose enhances the proteasome-dependent degradation of EIN3, whereas ethylene enhances its stability. The glucose-induced degradation of the EIN3 transcription factor is dependent on HXK1, as it is no longer detected in the AtHXK1-null mutant *gin2* (Yanagisawa et al., 2003).

Several reports have provided evidence for sugar interactions with other phytohormones, such as auxins, cytokinins, gibberellic acid (GA), salicylic acid (SA) and brassinosteroids (BR) (reviewed in Ramon et al., 2008). For instance, further phenotypical analyses of the *gin2* mutant have established sugar interactions with both

auxins and cytokinins. In fact, both the auxin resistant mutants *axr1, axr2* and *tir1* and constitutive cytokinin signaling mutants are insensitive to high concentrations of glucose (Moore et al., 2003). Glucose has also been shown to affect GA-mediated α-amylase expression in barley embryos, and several studies indicate that glucose and GA responses share the same promoter elements in rice (Perata et al., 1997; Morita et al., 1998; Chen et al., 2002). Finally, BR and SA are also linked to sugar signaling — the BR mutant *brassinosteroids, light and sugar1* (*bls1*) is hypersensitive to sugar, and sucrose- and glucose-induced expression of *PR* genes is abolished in mutants with reduced SA levels (Laxmi et al., 2004; Thibaud et al., 2004).

1.3.3.3. Sugars and nitrogen metabolism

Carbon (C), in the form of photosynthate, and nitrogen (N) are two of the most important elements required for normal plant growth and development. Carbohydrates provide both the energy and the carbon skeletons for nitrate and ammonium assimilation during amino acid biosynthesis, while amino acids and proteins are the key building blocks for the cell (Coruzzi and Bush, 2001). Therefore, cells must monitor both the status and balance between C and N to optimize their opportunity for metabolism, growth and development. As such, it is not surprising that C and N metabolism are tightly linked in almost every biochemical pathway in the plant. In fact, mutants with altered sensitivities to imbalanced C/N conditions have been isolated, providing identification of components in the C/N balance in plants. The *lateral root initiation1* (*lin1*) mutant, which has a mutation in a nitrate transporter gene (NRT2.1), shows reduced sensitivity to repressing environmental conditions (high C, low N) (Malamy and Ryan, 2001; Little et al., 2005), whereas the *oversensitive to sugar1* (*osu1*) mutant, deficient in a putative methyltransferase, shows enhanced anthocyanin accumulation and root inhibition under different variations of C/N conditions (Gao et al., 2008). Furthermore, the putative glutamate receptor 1.1 (GLR1.1) not only affects ABA biosynthesis, but also functions as a regulator of C and N metabolism in Arabidopsis (Kang and Turano, 2003).

Carbon metabolites are known to regulate genes involved in N acquisition and metabolism. Early studies on the *nitrate reductase* (*NR*) gene provided the first evidence for such regulation, as sugars were shown to affect both the transcription and activity of *NR* (Cheng et al., 1992; Crawford, 1995). Also, genes encoding diverse transporters for nitrogen and other nutrient uptake have been shown to be upregulated by sugars (Lejay et al., 1999; Lejay et al., 2003; Lejay et al., 2008), and conversely the expression of the nitrogen metabolism gene *asparagine synthase1* (*ASN1*) is repressed by sugars (Lam et al., 1998). In a global transcription profiling study in Arabidopsis, Price et al. (2004) observed that genes associated with nitrogen assimilation and amino acid metabolism were much more profoundly regulated by glucose than by nitrogen. Another microarray study focused on the Arabidopsis root system has identified molecular networks, among which are genes involved in metabolic pathways, protein degradation and auxin signaling, to be regulated by C, N or C/N interactions (Gutierrez et al., 2007).

1.3.3.4. Sugars and other components

One of the most common mechanisms in the regulation of signal transduction is protein phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases, respectively. A first indication of the involvement of phosphatases in sugar signaling came from the use of phosphatase inhibitors that were able to mimic sugar-induced repression of photosynthesis genes in maize protoplasts (Sheen, 1993) or block sucrose-induced expression of storage-related genes in sweet potato (Takeda et al., 1994). On the other hand, inhibition of protein kinases by application of staurosporine has been shown to enhance the glucose induction of an extracellular invertase (CIN1) and of phenylalanine ammonia-lyase (PAL) (Ehness et al., 1997). Such studies using phosphatase and/or protein kinase inhibitors have come in support of the role of phosphatases and protein kinases in sugar signaling and suggest the involvement of different phosphorylation mechanisms in glucose activation (reviewed in Rolland et al., 2002 and Ramon et al., 2008). Furthermore, an early report by Ohto

and Nakamura (1995) showed the involvement of Ca^{2+} signaling in the sugar-induction of the sporamin and β-amylase genes in sweet potato, pointing to an interaction between Ca^{2+}-mediated signaling and sugar signaling pathways.

1.3.4. Energy and stress sensing and signaling

Plants are constantly confronted with multiple types of stress that affect the plant's overall energy status. Stress is often associated with a reduction in photosynthesis and/or respiration, which in turn results in energy deprivation and ultimately in growth arrest and cell death (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). Metabolic and structural modifications caused by energy deficit are accompanied by changes in gene expression. Large-scale transcriptome profiling has revealed that the effect of sugar deprivation or prolonged darkness impacts more than a thousand gene targets (reviewed in Baena-Gonzalez and Sheen, 2008).

In order to cope with environmental challenges, organisms have evolved mechanisms that allow them to control energy and metabolic homeostasis. For instance, the sucrose non-fermenting1 (SNF1) protein kinase, a major component of yeast sugar signaling, is activated in response to glucose starvation, causing derepression of a large number of genes required for catabolism of alternative carbon sources and therefore ensuring sufficient ATP synthesis (Carlson, 1999; Hedbacker and Carlson, 2008). In mammals, a similar mechanism for glucose starvation involves the AMP-activated protein kinase, AMPK, which is activated by the increase in the ratio of AMP to ATP and is responsible for switching off ATP-consuming processes while activating ATP-generating catabolic pathways (Hardie, 2007). Homologous protein kinases, which belong to the family of SnRKs (SNF1-Related Protein Kinases), have been identified in plants. This protein family can be divided into the SnRK1, -2, and -3 sub-groups. The SnRK1 proteins are the most closely related to yeast SNF1 and mammalian AMPK, while the SnRK2 and SnRK3 groups are probably unique to plants (Halford et al., 2003). In Arabidopsis, there are three members of the SnRK1 subfamily but only two, SnRK1.1 (also known as KIN10) and SnRK1.2 (also known as KIN11),

are expressed (Bhalerao et al., 1999; Baena-Gonzalez et al., 2007). Recently, SnRK1.1 and SnRK1.2, collectively called SnRK1, have been shown to perform a similar task in Arabidopsis as SNF1 in yeast and AMPK in mammals (Baena-Gonzalez et al., 2007).

The identification of plant SnRK1 substrates and regulated genes provided the first evidence that these protein kinases are involved in the control of carbon and nitrogen metabolism in plants. In fact, three important Arabidopsis enzymes, 3-hydroxyl-3-methylglutaryl (HMG)-CoA reductase, sucrose phosphate synthase (SPS) and nitrate reductase (NR), involved in isoprenoid synthesis, sucrose biosynthesis and nitrogen assimilation, respectively, have been shown to be phosphorylated by SnRK1, leading to their inactivation (Halford et al., 2003; Rolland et al., 2006). Regarding SnRK1-specific target genes, a few were initially identified, including a potato (*Solanum tuberosum*) sucrose synthase gene activated by sucrose (Purcell et al., 1998) and a wheat α-amylase gene repressed by glucose (Laurie et al., 2003), as well as a rice α-amylase gene promoter MYBS1 (v-myb avian myeloblastosis viral oncogene homolog involved in sugar signaling) that was shown to be activated by SnRK1 under glucose starvation (Lu et al., 2007; reviewed in Baena-Gonzalez and Sheen, 2008). However, the regulation of gene expression by SnRK1 is not restricted to a few genes. In fact, a 2007 study in Arabidopsis has reported SnRK1 activation to trigger changes in the expression of over 1000 genes that allow the re-establishment of homeostasis by repressing energy-consuming processes such as biosynthetic pathways and promoting catabolic processes and photosynthesis to increase ATP generation (Baena-Gonzalez et al., 2007). In addition, the transcriptional profile induced by SnRK1.1 activity largely overlaps with the profiles obtained under dark and starvation conditions, but is the exact opposite of the profiles obtained from sucrose- or glucose-fed seedlings (Baena-Gonzalez et al., 2007; Baena-Gonzalez and Sheen, 2008). These findings provided evidence for SnRK1 as a central regulator of metabolism, functioning in stress and energy sensing/signaling as a global regulator of transcription networks (Figure 1.6).

The characterization of transgenic and mutant SnRK1 plants has provided important information on the physiological role of these protein kinases in plant growth

and development. Transgenic plants overexpressing SnRK1.1 display delayed senescence and flowering, altered flower architecture under long day conditions, and enhanced tolerance to nutrient-deprivation conditions, which is in agreement with a role for SnRK1.1 in stress response (Baena-Gonzalez et al., 2007). On the other hand, silencing of both *SnRK1.1* and *SnRK1.2* confers dramatic growth defects, such as senescence occurring before the onset of flowering (Baena-Gonzalez et al., 2007).

More recent studies have highlighted SnRK1 function as a central integrator of sugar signaling pathways, which is obviously interconnected with the energy and metabolic status of the plant. A recent report by Zhang et al. (2009) has implicated SnRK1 in the trehalose signaling pathway. In addition, Arabidopsis transgenic lines overexpressing SnRK1.1 are hypersensitive to both glucose and ABA, displaying also modification in the expression of the pathogenesis-related genes *PR1, PR2,* and *PR5* genes in response to glucose (Jossier et al., 2009). This suggests implication of the sugar signaling function of SnRK1.1 in the metabolism-dependent pathway of HXK1, potentially via ABA. The overexpression of SnRK1.1 also led to modifications in the content of soluble sugars, starch and AGPase activity on source tissues (leaves) in response to glucose (Jossier et al., 2009).

Protein kinases often function in cascades where upstream kinases activate downstream ones by phosphorylation. Indeed, similarly to SNF1 in yeast and AMPK in mammals, SnRK1 kinase activity is controlled by phosphorylation of a conserved threonine residue in the activation- or T-loop of its kinase domain (Hardie, 2007; Hedbacker and Carlson, 2008; Baena-Gonzalez, 2009; Shen et al., 2009). Consistent with a role in energy-depleting conditions, sugar phosphates (especially glucose-6-phosphate) have been shown to inhibit SnRK1 activity (Toroser et al., 2000). However, there is some controversy as to whether sugars act as activators or inhibitors of SnRK1 (reviewed in Baena-Gonzalez and Sheen, 2008). A very early report, in 1999, showed the activity of SnRK1.1 in protein extracts from Arabidopsis shoots and roots to be stimulated by sucrose (Bhalerao et al., 1999). Jossier et al. (2009), who have also observed the activity of SnRK1.1 to increase in response to glucose, suggest that SnRK1 kinases, as global regulators of plant metabolism, could respond

to changes in carbohydrate level, both deprivation and excess. Also, the fact that SnRK1.1 is included in a heterotrimeric complex means that various non-catalytic subunits could be available depending on the conditions, the tissue or the cellular compartment, allowing SnRK1.1 to respond to various stimuli (Jossier et al., 2009). Additionally, the discrepancies in activity of SnRK1 in response to sugars could also be due to different growth conditions, long-term sugar depletion, stress signaling and/or difficulties in measuring overt changes in SnRK1 activities (Baena-Gonzalez and Sheen, 2008).

Only a few components of the SnRK1 signaling cascade have been identified so far. Of particular importance is the Arabidopsis S-group of bZIP G-box binding transcription factors (bZIP1, bZIP2/GBF5, bZIP11, bZIP44, and bZIP53), which bind to the promoters of genes regulated by the SnRK1.1 signaling pathway (Baena-Gonzalez et al., 2007; Hanson et al., 2008). Interestingly, these transcription factors are repressed by sugars, which is also in agreement with an antagonistic regulation of the system by energy deficiency and energy abundance (Baena-Gonzalez, 2009).

In plants, SnRK1s interact with several proteins. Yeast two-hybrid assays have shown that pleiotropic regulatory locus1 (PRL1), a nuclear WD (TrpAsp) repeat protein, binds Arabidopsis SnRK1.1 and SnRK1.2, with PRL1 inhibiting their protein kinase activity in vitro (Bhalerao et al., 1999). Another WD40-containing protein, 5PTase13, a myoinositol polyphosphate 5-phoshatase, also interacts specifically with SnRK1.1 in vitro and is required to maintain wild-type levels of SnRK1.1 activity under low nutrient or high sugar conditions, while under starvation it negatively regulates the activity of SnRK1.1 (Ananieva et al., 2008). The decrease in kinase activity is correlated with a higher level of SnRK1.1 degradation by the proteasome (Ananieva et al., 2008). Furthermore, PRL1 has been reported to act as the substrate receptor for the degradation of SnRK1.1 by a CUL4 (cullin4) E3 ligase in Arabidopsis (Lee et al., 2008). In this study, the in vitro degradation of SnRK1.1 was found to be slower in protein extracts of the *prl1* and *cul4* mutants, with SnRK1.1 levels in the wild type being lower than those in *cul4* and *prl1* seedlings, indicating that both PRL1 and CUL4 are needed for SnRK1.1 degradation (Lee et al., 2008). These results led to the

hypothesis that PRL1 may be delivering SnRK1.1 to the CUL4-DDB1 complex for proteasomal degradation. Therefore, the absence of the PRL1-CUL4 E3 ubiquitin ligase results in increased amounts of SnRK1.1 in the *prl1* and *cul4* mutants, which could largely account for the reduction in growth, accumulation of anthocyanin, and ABA, cytokinin, and sugar hypersensitivity phenotypes displayed by these mutants (Lee et al., 2008). Interestingly, mutations in the *5PTase13* gene lead to an opposite phenotype — *5PTase13* mutants are insensitive to ABA and sugars (Ananieva et al., 2008). This suggests that under low-nutrient and sugar stress conditions, in which 5PTase13 acts as a positive regulator of SnRK1.1 activity, PRL1 and 5PTase13 have opposing functions (Ananieva et al., 2008), supporting the notion of PRL1 and/or 5PTase13 forming complexes with SnRK1.1, which in turn would regulate seedling responses to various nutrient and stress conditions.

1.3.5. mRNA metabolism-related genes involved in sugar or ABA signaling

A role for mRNA metabolism in the control of sugar and ABA signaling is supported by a few previous studies, which are briefly described below.

The *low-beta-amylase1* (*lba1*) mutant, originally isolated as a mutant showing reduced sugar-induced expression of the β-*amylase* (Atβ-*amy*) gene, reduced sugar-induced accumulation of anthocyanins and decreased chlorophyll content (Mita et al., 1997), was found to contain a missense mutation in the UP-Frameshift1 (AtUPF1) RNA helicase (Yoine et al., 2006). The UPF1 gene plays a central role in NMD via its RNA-binding helicase activities (Czaplinski et al., 1995). RNA helicases, highly conserved enzymes that modulate the structure of RNA, participate in all biological processes involving RNA, such as transcription, splicing and translation. In particular, helicases are important for RNA constitutive and alternative splicing regulation, being implicated in the correct recognition of RNA during spliceosome formation, rearrangement of the spliceosome, and regeneration of snRNPs between rounds of splicing (Honig et al., 2002). The *lba1* mutation causes hypersensitivity of seed germination to glucose and ABA, as well as a reduction in sugar-induced expression of

several genes other than *β-amy*, such as genes involved in senescence, nutrient storage and defensive reactions (Yoine et al., 2006). Based on these results, Yoine *et al.* (2006) suggested that UPF1-dependent posttranscriptional regulation is involved in sugar signaling being required for maximum expression of a subset of sugar-inducible genes (Yoine et al., 2006).

In 2001, Xiong et al. described a novel Arabidopsis mutant impaired in both ABA response and drought-induced ABA biosynthesis, the *sad1* (*super-sensitive to ABA and drought1*) mutant. The *SAD1* sequence was found to encode an Sm-like (Lsm) snRNP protein. Such proteins have been identified as structurally related to Sm proteins, a family of small proteins that assemble the core component of spliceosomal snRNPs present in all eukaryotes and prokaryotes (Salgado-Garrido et al., 1999). As an Lsm protein, SAD1 is most likely involved in different aspects of mRNA metabolism such as splicing, export and degradation (Xiong et al., 2001).

More recently, a gene encoding a conserved splicing factor was also implicated in ABA signaling. Suppressor of ABI3-5 (SUA) is an RNA-binding protein with two RRMs that interacts with the U2AF pre-spliceosomal component (Sugliani et al., 2010). On the other hand, the *Abscisic Acid Insensitive3* (*ABI3*) gene is a major regulator of seed maturation, and mutations in *ABI3* lead to seed insensitivity to ABA during germination, desiccation intolerance and reduced longevity (Ooms et al., 1993). Sugliani et al. (2010) have shown that the pre-mRNA of the *ABI3* transcription factor possesses a cryptic intron that is alternatively spliced leading to the occurrence of two transcripts, one encoding the full-length protein and the other a truncated form. Functional analyses in ABI3-5 and SUA-1 loss-of-function mutants indicate that SUA influences seed maturation by suppressing splicing of the cryptic *ABI3* intron. Higher abundance of SUA will favor cryptic intron retention and increase full-length ABI3 protein levels during seed maturation.

1.4. Thesis outline

The work presented in this thesis examines the regulation of Arabidopsis SR-related splicing factors at the transcriptional level and describes the functional and molecular characterization of the plant-specific SR45, showing it is involved in sugar and stress signaling in *Arabidopsis thaliana.* The thesis is comprised of five chapters, including this introductory Chapter 1. The remaining four chapters are structured as follows:

Chapter 2 – *Arabidopsis SR proteins: gene expression patterns and loss-of-function mutants.*

This chapter provides a global analysis of the tissue- and stress-specific expression and splicing patterns of the 18 members of the *Arabidopsis thaliana* SR protein family, as well as of the non-canonical SR protein gene, *SR45*. It also reports the isolation of genetic null mutants for four Arabidopsis SR proteins, as well as the isolation of a loss-of-function mutant for SR45, *sr45-1.*

Chapter 3 – *The plant-specific SR45 protein negatively regulates glucose and ABA signaling during early seedling development in Arabidopsis*.

This chapter presents the functional characterization of the *sr45-1* mutant, addressing the altered sugar and stress responses during early seedling development induced by depleted levels of the SR45 splicing factor.

Chapter 4 – *The Arabidopsis SR45 splicing factor, a negative regulator of sugar signaling, targets alternative splicing of the 5PTase13 gene and modulates SnRK1.1 levels.*

This chapter illustrates the mode of action of SR45 by assessing its involvement in specific sugar signaling pathways. Importantly, it also identifies splicing targets of this RNA-binding protein.

Chapter 5 – *Concluding remarks and future perspectives*

This chapter summarizes and discusses the major findings of the work presented in this thesis, suggesting future experimental follow-ups.

Chapter 3 is the reproduction of the following publication:

Carvalho, R., Carvalho, S., and Duque P. 2010. The plant-specific SR45 protein negatively regulates glucose and ABA signaling during early seedling development in Arabidopsis. Plant Physiology **154(2)**:772-783.

Chapter 4 constitutes a substantial part of a manuscript currently under preparation.

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CHAPTER 2

Arabidopsis SR proteins: gene expression patterns and loss-of-function mutants

2.1. Abstract

Members of the highly conserved serine/arginine-rich (SR) family of splicing factors participate in RNA-protein and protein-protein interactions, recruiting other factors during spliceosome assembly. SR proteins influence splice site selection in a concentration-dependent manner and are established key players in mammalian alternative splicing. At least 18 SR proteins are present in the Arabidopsis genome. Here, we have analyzed the tissue- and stress-specific expression and splicing patterns of the members of the *Arabidopsis thaliana* SR protein gene family, as well as of the non-canonical SR protein gene, *SR45*, which has been recently excluded from the Arabidopsis SR protein family. The majority of these genes are detected in seeds, imbibed seeds, roots, leaves and flowers, with highest expression levels being detected in flowers. Moreover, four SR genes display alternative splicing transcripts. In general, cold stress and the application of the stress phytohormone abscisic acid (ABA) modulate SR protein gene expression, with the splicing pattern of two SR genes being altered by cold. Expression of the *SR45* gene is affected by ABA, high salinity and temperature stress. Taken together, our results suggest an involvement of these splicing factors in plant responses to abiotic stress. A future major challenge will be to uncover the precise in vivo function of each Arabidopsis SR-related protein. To this end, we have isolated genetic null mutants for *SR34, SCL30a*, *SCL33* and *RS31*. Future phenotypical analyses, particularly under different stress conditions, will provide invaluable clues on the mode of action of this important class of splicing regulators. Finally, we report the isolation of a loss-of-function mutant for *SR45*, whose functional characterization is reported in the following chapters of this thesis.

2.2. Introduction

SR (serine/arginine-rich) proteins are members of a highly conserved family of structurally and functionally related spliceosomal proteins, which have been shown in animal systems to play vital roles in both constitutive and alternative splicing by

influencing the most crucial steps of spliceosome assembly (Wu and Maniatis, 1993; Kohtz et al., 1994; Manley and Tacke, 1996; Shen and Green, 2004).

The members of this family of essential splicing factors share a characteristic structural organization, containing one or two RNA-recognition motifs (RRMs) at their N-termini that provide RNA-binding specificity, and a reversibly phosphorylated arginine/serine-rich (RS) domain at their C-termini that acts to promote protein-protein interactions. The RS domain also functions in promoting pre-spliceosome assembly by contacting directly with the pre-mRNA via the branch point and the 5' splice site (Shen and Green, 2004; Shen et al., 2004; Hertel and Graveley, 2005). By binding to specific RNA binding sites such as exonic splicing enhancers (ESEs), SR proteins are able to recruit other spliceosomal proteins such as U2AF or the U1 snRNP to weak splice sites and promote splicing. They have also been shown to play crucial roles in alternative splicing by determining splice site selection in a concentration-dependent manner (Graveley, 2000). In addition to their roles in precursor-mRNA (pre-mRNA) splicing, these RNA-binding proteins also function in mRNA transport (Huang and Steitz, 2001), mRNA stability and mRNA nonsense-mediated decay (NMD) (Zhang and Krainer, 2004) as well as translation (Sanford et al., 2004). They have also been reported to function in coupling transcription to splicing, by associating on nascent pre-mRNAs (Das et al., 2007). More recently, the mammalian ASF/SF2 SR protein was shown to be involved in the regulation of protein sumoylation, stimulating SUMO conjugation (Pelisch et al., 2010).

The existence of SR proteins in plants was first shown by screening protein extracts from Arabidopsis, tobacco and carrot with antibodies raised against a conserved phosphoepitope located in the RS domain (Lazar et al., 1995; Lopato et al., 1996a). Several of these plant phosphoproteins have been shown to complement inactive HeLa cell cytoplasmic splicing extracts and to be active in heterologous alternative splicing assays (Lazar et al., 1995; Lopato et al., 1996a; Lopato et al., 1996b; Lopato et al., 1999b; Ali et al., 2007; Barta et al., 2008), suggesting conserved functions of SR proteins in splicing among plants and metazoans.

Among eukaryotes, flowering plants have the highest number of SR proteins with a total of 24 in rice (Iida and Go, 2006), 17 in Brachypodium (International Brachypodium Initiative, 2010) and 18 in Arabidopsis (Barta et al., 2010), whilst there are only seven SR genes in *C. elegans* (Longman et al., 2000; Manley and Krainer, 2010) and 12 in humans (Manley and Krainer, 2010). Genome amplification, particularly gene duplication events, most likely explains the larger and more diverse families of these proteins in plants. In fact, at least 12 of the Arabidopsis SR genes are located on duplicated segments of the genome (Kalyna and Barta, 2004).

The family of SR proteins in Arabidopsis can be divided into six subfamilies, three of which (SR, RSZ and SC) are constituted by orthologs of human SR proteins (Figure 2.1). The other three subfamilies (RS, RS2Z and SCL) are plant-specific, including members with a unique domain organization not found in any metazoan organism (Figure 2.1) (Barta et al., 2008; Barta et al., 2010). Members of the RS subfamily possess an RS domain highly enriched in arginines rather than serine-arginine dipeptides, and contain two typical RRM domains without the SWQDLKD signature in their second RRM, which is characteristic of ASF/SF2-like proteins of the SR subfamily (Figure 2.1) (Lopato et al., 1996b; Barta et al., 2008; Barta et al., 2010). In contrast to the homologs of the human 9G8-like proteins from the RSZ subfamily, SR proteins belonging to the RS2Z plant-specific subfamily contain an RRM, two zinc knuckles and an SR domain followed by a domain rich in serines and prolines (Figure 2.1) (Lopato et al., 2002; Barta et al., 2008; Barta et al., 2010). Finally, members of the SCL subfamily, similarly to the SC35 protein from the SC subfamily, contain a single RRM followed by an RS domain, but possess in addition a short N-terminal extension rich in arginines, prolines, serines, glycines and tyrosines (Figure 2.1) (Golovkin and Reddy, 1999; Barta et al., 2008; Barta et al., 2010).

Subfamily	Gene/Protein Symbol	Gene Locus
Mammalian orthologs		
SR ASF/SF2 (SRSF1) orthologs	SR30 SR34 SR34a SR34b	At1g09140 At1g02840 At3g49430 At4g02430
RSZ 9G8 (SRSF7) orthologs	RSZ21 RSZ22 RSZ22a	At1g23860 At4g31580 At2g24590
SC SC35 (SRSF2) orthologs	SC35	At5g64200
Plant-specific		
SCL	SCL28 SCL30 SCL30a SCL33	At5g18810 At3g55460 At3g13570 At1g55310
RS2Z	RS2Z32 RS2Z33	At3g53500 At2g37340
RS	RS31 RS31a RS40 RS41	At3g61860 At2g46610 At4g25500 At5g52040

Figure 2.1. Schematic representation of the Arabidopsis SR protein gene family

Structural features and subfamilies of Arabidopsis SR proteins. Nomenclature and classification recently proposed by Barta *et al.* (2010). Abbreviations: RRM, RNA-recognition motif; *RRM*, RRM that contains the SWQDLKD motif, which is present in all ASF/SF2 homologs; SR, domain rich in serine-arginine dipeptides; RS, domain rich in arginines and serines; ZnK, zinc knuckle of CCHC type; GH, glycine hinge; SP, domain rich in serines and prolines; PSK, region rich in proline, serine and lysine; SCL proteins contain an N-terminal extension rich in arginines, prolines, serines, glycines and tyrosines (white box).

Subcellular localization studies for the large majority of plant SR proteins have shown that they are restricted to the nucleus – diffusely distributed in the nucleoplasm or accumulated in speckles, depending on the developmental stage, the cell's type, cell cycle phase or physiological state, as well as the phosphorylation status of the SR protein (Docquier et al., 2004; Fang et al., 2004; Lorković et al., 2004; Tillemans et al., 2005; Tillemans et al., 2006; Ali and Reddy, 2008). Speckles are defined as subnuclear structures, corresponding to interchromatin granules, that may act as storage, assembly and/or modification sites from which splicing factors are recruited to regulate splicing at the transcription sites (Misteli et al., 1997). Plant SR proteins from different subfamilies localize to different populations of nucleoplasmic speckles,

suggesting that different SR proteins interact with different subsets of transcripts to effect the splicing, alternative splicing, or transport of pre-mRNAs or mRNAs (Shaw and Brown, 2004; Lorkovic et al., 2008). A recent study has demonstrated that RS2Z22 is a bona fide nucleocytoplasmic shuttling SR protein (Rausin et al., 2010), indicating that this protein could be involved in post-splicing activities, such as mRNA nuclear export.

Arabidopsis SR proteins have been shown to interact directly with other SR proteins as well as with protein kinases, cyclophilins and spliceosomal proteins (Reddy, 2007; Barta et al., 2008). In particular, SCL33, RSZ21, RSZ22 and SR34 interact with U1-70K, one of the U1 snRNP specific proteins implicated in regulating basic splicing and alternative splicing of pre-mRNAs (Golovkin and Reddy, 1996, 1998, 1999; Lorkovic et al., 2004).

A few plant SR proteins have been shown to regulate splice site choices in vivo (Lazar and Goodman, 2000; Kalyna et al., 2003; Gao et al., 2004; Isshiki et al., 2006; Lopato et al., 2006; Ali et al., 2007). Also, the majority of SR protein genes are themselves alternatively spliced and some of the alternative splicing events are evolutionarily conserved, suggesting that their regulation by alternative splicing is important for plant development (Kalyna et al., 2006; Palusa et al., 2007). Interestingly, the alternative splicing of several SR genes is significantly altered by different forms of abiotic stress, including temperature stress (Lazar and Goodman, 2000; Palusa et al., 2007; Filichkin et al., 2010), high salinity (Palusa et al., 2007; Tanabe et al., 2007) and high light irradiation (Tanabe et al., 2007; Filichkin et al., 2010). This way, stress-induced changes in SR protein gene products could alter the splicing of different downstream targets resulting in adaptive transcriptome changes in response to environmental cues (Ali and Reddy, 2008; Duque, 2011). Stress signals are also known to control both the phosphorylation status and the subcellular localization of plant SR proteins. An example is RSZ22, reported to display a specific nucleolar concentration upon phosphorylation inhibition and experimental stress imposed by long observation periods that lead to a continuous decrease of cellular ATP level (Tillemans et al., 2006; Rausin et al., 2010).

As mentioned above, SR genes undergo alternative splicing themselves. Indeed, it appears that 95 transcripts are produced from 14 Arabidopsis SR genes, thereby increasing the transcriptome complexity generated by this gene family by six-fold (Palusa et al., 2007). Although the functional significance of most of the SR protein alternative transcripts is not known, it was found that many result in the inclusion of a premature stop codon (PTC) (Palusa et al., 2007), therefore rendering them potential targets for NMD, an mRNA surveillance mechanism that functions to prevent accumulation of truncated and potentially harmful proteins. A recent study has shown that about half of these alternative SR transcripts are indeed targeted for degradation by NMD and could consequently provide a way of fine-tuning the abundance of functional transcripts and thereby the abundance of SR proteins (Palusa and Reddy, 2010).

The Arabidopsis SR45 protein has been regarded as a classical SR protein because it could complement an animal in vitro splicing extract deficient in SR proteins (Ali et al., 2007). However, due to its highly atypical SR protein structure — two RS domains separated by an RRM — it now falls outside the very recently proposed definition of SR proteins (Barta et al., 2010).

We have performed an analysis of the expression and splicing patterns of all 18 Arabidopsis SR genes as well as of *SR45* in different plant tissues and in response to cold stress and to the exogenous application of the stress phytohormone abscisic acid (ABA). For the *SR45* gene, the effect of other environmental stresses (heat, high salinity and drought) was also examined. Our results show that *SR45* as well as all members of the Arabidopsis SR protein gene family are constitutively expressed in roots and flowers, showing highest expression levels in the latter organ. Expression of *SR45* and of all SR genes except for *SCL28* and *SCL33* was also detected in leaves. Although SR transcript levels were barely detectable in dry seeds, gene expression was induced by seed imbibition for *SR45* and more than half of the Arabidopsis SR proteins. In general, expression of *SR45* and the SR protein genes was altered by stress and ABA. Interestingly, the splicing pattern of *SR30* and *SR34* was altered by cold stress. Finally, we report the isolation of a homozygous loss-of-function mutant for

each of eight SR protein genes, four of which represent genetic null mutants, as well as three homozygous alleles for *SR45*.

2.3. Results

2.3.1. Tissue- and stress-specific gene expression and splicing patterns

To initiate the characterization of *Arabidopsis thaliana* SR proteins and establish a framework for future analysis of the function of these RNA-binding proteins particularly in plant responses to environmental cues, tissue- and stress-specific expression and splicing patterns were examined for the 18 members of the Arabidopsis SR protein gene family as well as for the non-canonical SR protein gene, *SR45*, which has been recently excluded from the Arabidopsis SR protein family (Barta et al., 2010).

To examine transcript levels by RT-PCR, in early 2007 gene-specific primers (Table 2.1) were designed to allow for distinction of the splice variants described in "The Arabidopsis Information Resource" (TAIR) website (http://www.arabidopsis.org). However, with gene structures being updated one-two times per year, the designed primers are not able to detect a few new splice variants, which have subsequently been included in the TAIR database and/or reported by Reddy and co-workers (Table 2.2) (Ali et al., 2007; Palusa et al., 2007).

Regarding the stress-specific expression splicing patterns, and to ensure that all stress treatments were effective, RT-PCR analyses were first performed with primers specific to genes whose expression is known to be altered by the external signals in question, namely *RD29A* induced by ABA, NaCl, cold and drought (Ishitani et al., 1997), *HSP18* induced by heat shock (Lohmann et al., 2004; Price et al., 2004) and *RBCS* repressed by glucose (Cheng et al., 1992) (Figure 2.2, Table 2.3). The *18S* and *cyclophylin* housekeeping genes were used as loading controls (Table 2.3).

Table 2.1. Gene-specific primers used for the analysis of tissue- and stress-specific expression and splicing patterns of Arabidopsis SR protein genes and the *SR45* gene.

Primers annealing in exonic sequences allowing distinction of the splice variants described in the TAIR website in early 2007. Except where indicated, all exons are in the open-reading frame (ORF).

Gene Name	Locus ID	Primers	Location	Product sizes (bp)
SR30	At1g09140	Fwd: 5'-ATGAGTAGCCGATGGAATCG-3'	Exon1	**758 / 1200**
		Rev: 5'-CAGGTGAAACTGGAGAATTCG-3'	Exon 11	
SR34	At1g02840	Fwd: 5'-GAAGGTTCCTCCAAGGCC-3'	Exon 2	**706 / 1060**
		Rev: 5'-GAGATCTTGATCTTGAACGCG-3'	Exon 10	
SR34a	At3g49430	Fwd: 5'-TTGGATGGCTGTCGCTTGAGG-3'	Exon 3	**263**
		Rev: 5'-CTGTCTCGAGTCACCTCAGC-3'	Exon 6	
SR34b	At4g02430	Fwd: 5'-CATCTGCGTCCTGGCAAGACC-3'	Exon 5	**153 / 163***
		Rev: 5'-CGAAACTCTGTGTCATCGAGC-3'	Exon 8	
RSZ21	At1g23860	Fwd: 5'-AGTGCTATGAGTGTGGTGAGC-3'	Exon 3	**286**
		Rev: 5'-ATTGGCATATGGCGAACGTCG-3'	Exon 4	
RSZ22	At4g31580	Fwd: 5'-TACTGAGCGTGAACTTGAGG-3'	Exon 1	**519**
		Rev: 5'-ATATGGCACTTCCTCACGCG-3'	Exon 4	
RSZ22a	At2g24590	Fwd: 5'-ATCTCCTCCTCCTCCAAGACG-3'	Exon 4	**279**
		Rev: 5'-GCAAGCATATCCAATCCACC-3'	Exon 5	
SC35	At5g64200	Fwd: 5'-ATGTCGCACTTCGGAAGG-3'	Exon 1	**632**
		Rev: 5'-GGAACGACTGTGACTGCG-3'	Exon 6	
SCL28	At5g18810	Fwd: 5'-CGAATCCTCTGGTCCTTCTGG-3'	Exon 1	**416**
		Rev: 5'-CTTAAGGATCGAGAACGGG-3'	Exon 6	
SCL30	At3g55460	Fwd: 5'-ATGAGGAGATACAGTCCGC-3'	Exon 1	**457**
		Rev: 5'-ATGAGAACGATCCCTGGAGC-3'	Exon 4	
SCL30a	At3g13570	Fwd: 5'-TCTCTTGGTTCGCAACTTACG-3'	Exon 1	**480**
		Rev: 5'-GCCATTATGGGGTGGTGAGCG-3'	Exon 5	
SCL33	At1g55310	Fwd: 5'-TCGGGATAGAAGACGTACTCC-3'	Exon 4	**266**
		Rev: 5'-TATGCTTCTTCTAGGGCTGG-3'	Exon 5	
RS2Z32	At3g53500	Fwd: 5'-ATCGCTATGGAAACACTCGCC-3'	Exon 2§	**356 / 594**
		Rev: 5'-GTGTCCTCTTTCACCACAGCG-3'	Exon 3	
RS2Z33	At2g37340	Fwd: 5'-TAGGACCCGAGACCTTGAACG-3'	Exon 1	**326**
		Rev: 5'-TGTCCTCTCTCTCCACAACGG-3'	Exon 4	
RS31a	At2g46610	Fwd: 5'-ATGAGACATGTGTACGTTGGG-3'	Exon 1	**509**
		Rev: 5'-TTCACCAGCCTCCCTCAAGGC-3'	Exon 4	
RS31	At3g61860	Fwd: 5'-ATGAGGCCAGTGTTCGTCGG-3'	Exon 1	**557 / 1242**
		Rev: 5'-ATACACAGGACTAAGAGACC-3'	Exon 4	
RS40	At4g25500	Fwd: 5'-TCATCCAGCATGAGACCTTCC-3'	Exon 3	**600**
		Rev: 5'-TCGGACTCTCCTTCTTGAACG-3'	Exon 4	
RS41	At5g52040	Fwd: 5'-CTTGGAGAGGCACTTTGAGCC-3'	Exon 3	**548**
		Rev: 5'-TCCCTTCTCTTCTCATATGGC-3'	Exon 4	
SR45	At1g16610	Fwd1: 5'-AACGTTCACACTACCACCTCG-3'	Exon 6	**589 / 568***
		Rev1: 5'-GTAAGAAGATGACCTCCCACG-3'	Exon 10	
		Fwd2: 5'-GCTGACGCTGAGAAAGATGGTGGTC-3'	Exon 6	**223 / 244†**
		Rev2: 5'- TTGGAGGGGGAGAAGATGGAGAACG-3'	Exon 7	
		Fwd3: 5'-TCCACCTCCAAGGAGACTACG-3'	Exon 9	**157†**
		Rev3: 5'-GTAAGAAGATGACCTCCCACG-3'	Exon 10	

* Splice variants not easily separated in 1% agarose gels.

§ Exon located in the 5' UTR.

† Location of primers is shown in Figure 2.3.

Table 2.2. Summary of described alternative splicing events in *SR45* and SR protein Arabidopsis genes.

Comparison of the number of splice variants detected in this study to that described in the TAIR website until December 28, 2010 and by Palusa *et al.* (2007). Numbers corresponding to the individual splice variants are shown between parentheses. The gene-specific primers used in this study and the sizes of the expected products are shown in Table 2.1.

Gene Name	Locus ID	# splice variants described in Palusa *et al.* (2007)	# splice variants described in TAIR	# splice variants detected in this study
SR30	At1g09140	**5** (1,2,3,4,5)	**2** (1,4)	**2** (1,4)
SR34	At1g02840	**7** (1,2,3,4,5,6,7)*	**3** (1,4) (8)[§]	**2** (1,4)*
SR34a	At3g49430	**4** (1,2,3,4)	**3** (1,2,3)	**1***
SR34b	At4g02430	**9** (1,2,3,4,5,6,7,8,9)	**2** (4,6)	**1**[ʊ]
RSZ21	At1g23860	**1**[†]	**4** (1) (2,3,4)[§]	**1**[†]
RSZ22	At4g31580	**1***	**2** (1) (2)[§]	**1***
RSZ22a	At2g24590	**1**	**1**	**1**
SC35	At5g64200	**3** (1,2,3)	**2** (1,2)	**1***
SCL28	At5g18810	**1**	**1**	**1**
SCL30	At3g55460	**2** (1,2)	**1** (1)	**1** (1)
SCL30a	At3g13570	**5** (1,2,3,4,5)	**1** (1)	**1** (1)
SCL33	At1g55310	**7** (1,2,3,4,5,6,7)	**2** (1,2,3)	**1***
RS2Z32	At3g53500	**7** (1,2,3,4,5,6,7)	**2** (1,3)	**2** (1,3)
RS2Z33	At2g37340	**4** (1,2,3,4)	**3** (1,2,3)	**1** (1)
RS31a	At2g46610	**9** (1,2,3,4,5,6,7,8,9)	**2** (1,5)	**1** (1)
RS31	At3g61860	**8** (1,2,3,4,5,6,7,8)	**1** (1)	**2** (1,4)
RS40	At4g25500	**6** (1,2,3,4,5,6)	**4** (1,2,3) (7)[§]	**1***
RS41	At5g52040	**6** (1,2,3,4,5,6)	**4** (1,4) (7,8)[§]	**1***
SR45	At1g16610	**2** (1,2)	**3** (1,2) (3)[§]	**2** (1,2)

* Primers used do not allow detection of all reported splice variants.

§ Additional splice variants included in TAIR, but not described by Palusa *et al.* (2007).

† Primers used do not allow distinction between splice variants 1, 2 and 4.

ʊ Primers used allow detection of splice variants 1, 3, 4, 5, and 6, but sizes are too similar to be easily detected in 1% agarose gels.
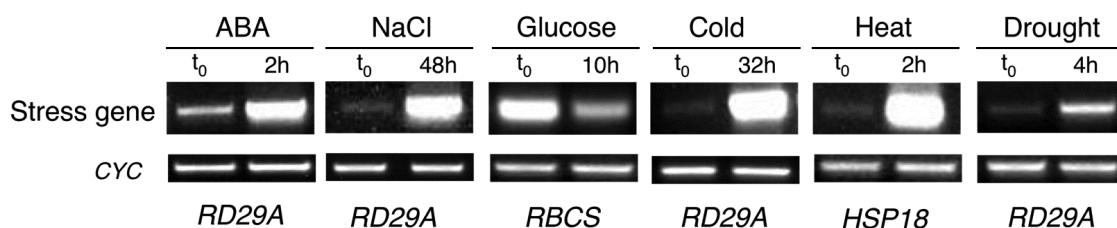
Figure 2.2. Verification of the effectiveness of stress treatments using established stress-responsive genes.

Two-week old seedlings were transferred to 30 μM ABA, 250 mM NaCl or 10% glucose, or placed at 4°C (cold stress) or 38°C (heat stress); for drought stress, three-week old seedlings were transferred to 3MM paper. Stress-responsive genes used were *RD29A (*At5g52310) induced by ABA, NaCl, cold and drought, *HSP18* (At5g59720) induced by heat shock, and *RBCS* (At5g38410) repressed by glucose. The *cyclophilin* (*CYC*) gene was used as a loading control. The sequences of the primers used in this RT-PCR analysis are shown in Table 2.3.

Table 2.3. Primers used to detect Arabidopsis stress-responsive and housekeeping genes.

Primers specific for three stress-responsive genes altered by ABA, NaCl and cold (*RD29A*), heat (*HSP18*) or glucose (*RBCS*) treatments as well as for two housekeeping genes (*18S* and *cyclophilin*).

Gene Name	Locus ID	Primers
RD29A	At5g52310	Fwd: 5'-GGAAGAGTCGGCTGTTTCAG-3'
		Rev: 5'-GTGCTCTGTTTTGGCTCCTC-3'
HSP18	At5g59720	Fwd: 5'-TCCAAGCATTTTTGGAGGAC-3'
		Rev: 5'-GAACCACAACCGTAAGCACA-3'
RBCS	At5g38410	Fwd: 5'-GGCTAAGGAAGTTGACTACC-3'
		Rev: 5'-ACTTCCTTCAACACTTGAGC-3'
18S	Ribosomal RNA	Fwd: 5'-CTGCCAGTAGTCATATGCTTGTC-3'
		Rev: 5'-GTGTAGCGCGCGTGCGGCCC-3'
cyclophilin	At4g38740	Fwd: 5'-GTCTGATAGAGATCTCACGT-3'
		Rev: 5'-AATCGGCAACAACAACAGGC-3'

2.3.1.1. Arabidopsis SR protein gene family

The expression levels of Arabidopsis SR protein genes were first analyzed by RT-PCR in different tissues of wild-type Col-0 plants, namely in dry seeds, imbibed seeds, roots, leaves and flowers. As seen in Figure 2.3A, all 18 SR protein genes appear to be constitutively expressed in roots and flowers. In leaves, expression of two SR

protein genes from the SCL subfamily, *SCL28* and *SCL33*, was not detected while *RS2Z33* and *RS31a* displayed very low transcript levels in this organ. With the exception of *SR30*, *SR34a*, *SCL30*, *RS31* and *RS40*, SR transcripts were barely detected in dry seeds, but seed imbibition induced the expression of more than half of the Arabidopsis SR protein genes (Figure 2.3A). All SR protein subfamilies showed highest expression in flowers with three members of different subfamilies, *SR34*, *RS2Z32* and *RS31a* showing high but similar expression levels in both flowers and roots (Figure 2.3A). Interestingly, *SR30*, *SCL30* and *RS40* displayed high levels of expression in both embryonic and vegetative tissues.

In this comprehensive analysis, only four SR protein Arabidopsis genes were found to undergo alternative splicing: *SR30*, *SR34*, *RS2Z32* and *RS31* (Figure 2.3, Tables 2.1 and 2.2). For the latter gene, although no splice variants are described in the TAIR database, it was possible to identify a new isoform, which has also been reported by Palusa *et al.* (2007) and referred to as isoform 4 (Figure 2.3, Tables 2.1 and 2.2). Splice variants were observed mainly in vegetative tissues, but an *SR30* alternative transcript could be detected, albeit at low levels, in imbibed seeds. The four genes found to undergo alternative splicing showed higher levels of the shorter transcript, which in all four cases encodes the full-length protein, than the longer transcripts encoding truncated proteins (Figure 2.3A). For *SR34* and *RS31*, the longer transcript showed highest expression in flowers, whereas for the remaining two SR proteins the longer splice variants were detected at lower and similar levels in the different organs (Figure 2.3A).
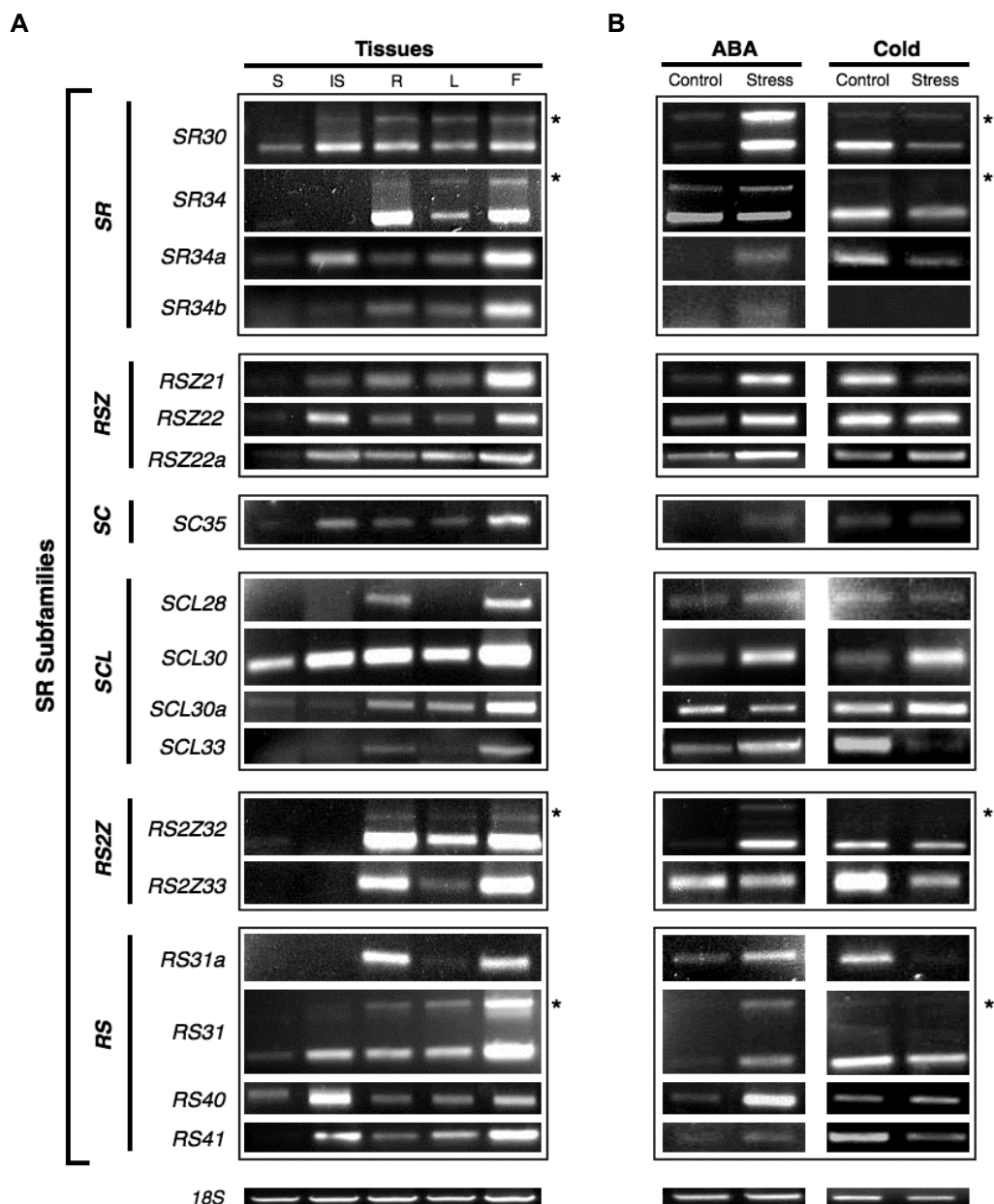
Figure 2.3. Tissue- and stress-specific expression and splicing patterns of the *Arabidopsis thaliana* family of SR protein genes.

A. RT-PCR analysis of transcript levels in dry (S) and imbibed (IS) seeds, as well as in roots (R), leaves (L) and flowers (F) of four-week old plants. **B.** RT-PCR analysis of transcript levels in the presence of ABA and under cold-stress conditions (two-week old seedlings were transferred to 30 μM ABA for 2h or placed at 4ºC for 32h). Asterisks indicate alternative transcripts. The *18S* gene was used as a loading control. The sequences of the primers used are shown in Tables 2.1 and 2.3.

To initiate the analysis of the effect of stress cues on the expression and splicing patterns of Arabidopsis SR protein genes, seedlings were either treated with the stress phytohormone ABA or exposed to cold stress (Figure 2.3B). Notably, 15 of the SR protein genes were found to be induced by the exogenous application of ABA, while two (*SCL30a* and *RS2Z33*) appeared to show slight downregulation by this phytohormone (Figure 2.3B). Interestingly, at the developmental stage at which the stress treatments were conducted, the longer transcript of two of the genes mentioned above to undergo alternative splicing, *RS2Z32* and *RS31,* could only be detected upon ABA application*.* Expression of *SR34* was unaffected by the ABA treatment (Figure 2.3B).

On the other hand, cold stress repressed the expression of eight SR protein genes, whereas five (*RSZ22*, *SC35*, *SCL28, RS2Z32*, *RS31*) were unaffected by low temperatures (Figure 2.3B). The two closely related genes encoding SC35-like proteins, *SCL30* and *SCL30a*, showed induction in response to cold (Figure 2.3B), while *RSZ22a* and *RS40* also appeared upregulated but to a lower extent. Interestingly, the longest splice variant of *SR34*, which encodes a truncated protein, was no longer detectable upon cold treatment. Cold stress also affected the splicing pattern of *SR30,* markedly downregulating the shortest transcript encoding the full-length protein but not the longest, which encodes a truncated version of SR30 (Figure 2.3B).

The developmental stage at which the stress treatments were carried out (two weeks after germination) only allowed detection of SR gene *SR34b* upon ABA treatment.

2.3.1.2. Arabidopsis SR45 gene

RT-PCR analysis of *SR45* transcript levels revealed ubiquitous expression of the gene in both embryonic and vegetative tissues. Although in embryonic tissues *SR45* expression could hardly be detected in dry seeds, the gene was markedly induced

after seed imbibition (Figure 2.4A). In vegetative tissues, expression was highest in flowers and lowest in roots (Figure 2.4A).
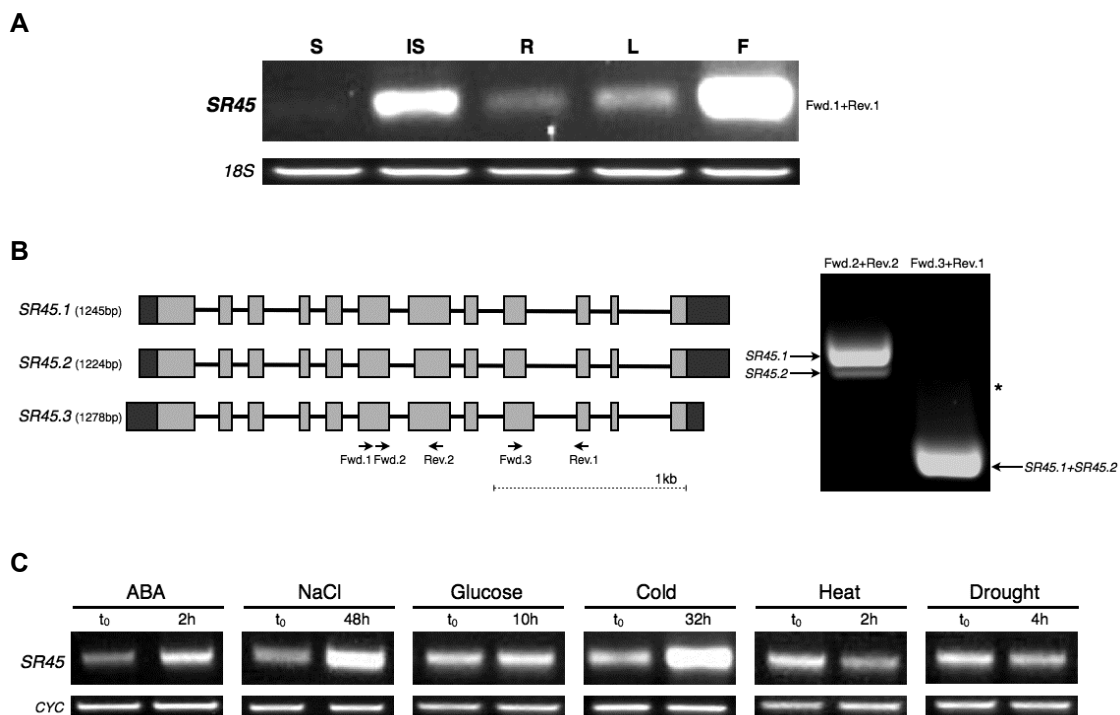
**Figure 2.4. Tissue- and stress-specific expression and splicing patterns of the Arabidopsis *SR45* gene.**

A. RT-PCR analysis of transcript levels in dry (S) and imbibed (IS) seeds, as well as in roots (R), leaves (L) and flowers (F) of four-week old plants. The *18S* gene was used as a loading control. **B.** Schematic representation of the three *SR45* alternatively-spliced transcripts, *SR45.1, SR45.2* and *SR45.3* (numbers between brackets indicate the size of the mature transcript). Rectangles represent exons (darker rectangles are exons in the UTRs) and lines represent introns. The sequences of the forward (Fwd.) and reverse (Rev.) primers indicated are shown in Table 2.1. **C.** RT-PCR analysis of the *SR45* splicing pattern in four-week old plants. Asterisk indicates the expected position of the *SR45.3* transcript. **D.** RT-PCR analysis of transcript levels under different stress conditions (two-week old seedlings were transferred to 30 μM ABA, 250 mM NaCl or 10% glucose; or placed at 4ºC or 38ºC for cold and heat stress respectively; for drought stress, three-week old seedlings were transferred to 3MM paper). The *cyclophilin* (*CYC*) gene was used as a loading control.

The *SR45* gene has been reported to generate two alternative transcripts (Ali et al., 2007; Palusa et al., 2007; Zhang and Mount, 2009), with splice variant 1 (*SR45.1*) including a 21-nucleotide sequence that is absent from splice variant 2 (*SR45.2*) (Figure 2.4B). Recently, in September 2010, the TAIR database included the annotation of a third isoform (*SR45.3*), which contains a 33-nucleotide intronic sequence that is absent from the other two splice isoforms (Table 2.2, Figure 2.4B). In *SR45.2*, an alternative 3' splice site in the sixth intron leads to the exclusion of 21 base pairs, while an alternative 5' splice site in the ninth intron results in the inclusion of 33 base pairs in *SR45.3*. Both alternatively-spliced segments are in frame, and hence none of the splice isoforms encode a truncated protein. As the three splice variants display very similar sizes, primers annealing close to the alternatively-spliced segments were designed and a higher concentration agarose gel was used in an attempt to separate them (Table 2.1, Figure 2.4B). As seen in Figure 2.4C, it was only possible to detect *SR45.*1 and *SR45.2,* with the former showing highest expression. SR45.3 was undetectable in the four-week old plant material analyzed.

To study the effect of stress signals on the expression and splicing pattern of *SR45*, two-week old seedlings were treated with ABA or exposed to different stresses such as cold, heat, high salinity and drought. In addition, seedlings were also treated with glucose, which has been shown to be a potent signal for transcriptional regulation, affecting a broad range of gene classes (Price et al., 2004). As shown in Figure 2.4D, *SR45* was found to be upregulated by ABA, salt and cold stresses, but downregulated under heat stress. The glucose and drought treatments did not affect expression of the Arabidopsis *SR45* gene.

2.3.2. Isolation of loss-of-function mutants

To initiate the functional analysis of individual Arabidopsis SR-related splicing factors, several loss-of-function mutants were isolated. To this end, T-DNA insertion lines were obtained from the SALK gene collection (http://signal.salk.edu) for the

Arabidopsis *SR45* and for 13 of the 18 SR protein genes (Table 2.4). For the remaining five SR genes, no T-DNA insertion mutants were yet available at the time the present study was initiated.

The mutant lines obtained were initially screened for kanamycin resistance, the selection marker for T-DNA insertions. As seen in Table 2.4, this resistance was silenced in several of the obtained lines, but when possible kanamycin selection was performed before PCR-based genotyping of individual plants. All SALK lines were genotyped using gene-specific primers flanking the insertion site reported in the SALK database and the T-DNA-specific primers LBc and LBd, annealing at the end of the left border (LB) of the T-DNA. This screen allowed the isolation of homozygous lines for insertions in eight Arabidopsis SR genes — *SR34* (At1g02840), *RSZ21* (At1g23860), *RSZ22a* (At2g24590), *SC35* (At5g64200), *SCL30a* (At3g13570), *SCL33* (At1g55310), *RS2Z33* (At2g37340) and *RS31* (At3g61860). A schematic diagram of each of these eight genes including the location of the T-DNA insertions is shown in Figure 2.5A. For the SALK lines with insertions in *SR30* (At1g09140), *SR34a* (At3g49430), *SR34b* (At4g02430), *SCL30* (At3g55460) and *RS41* (At5g52040), 16-20 plants were genotyped for each line but no plants homozygous for the insertion were retrieved.

For the eight homozygous SR mutant lines, genomic PCR using primers flanking the insertions (Table 2.4 and Figure 2.5A) amplified the expected gene size product for the Col-0 wild type, but not for any of the SR protein mutants (Figure 2.5B), indicating gene disruption in all lines. By contrast, PCR using the LBc or LBd primers (Table 2.4, Figure 2.5A) amplified a product in the mutant lines but not in Col-0, confirming the presence of the insertion in each mutant (Figure 2.5B). The resulting LB-specific fragments were then sequenced to verify the exact insertion location.

Table 2.4. SALK T-DNA insertion lines and corresponding gene-specific primers used for mutant line genotyping and analysis of transcript levels.

T-DNA insertion lines for the Arabidopsis *SR45* and 13 of the 18 SR protein genes obtained from the SALK collection. Kanamycin resistance and gene-specific primers, flanking the reported SALK T-DNA insertion site, as well as the T-DNA specific primers, LBc and LBd, are shown. Primers used for RNA analysis flanked the T-DNA insertion, except for SALK_083782 and SALK_018237 (T-DNA located in UTRs). The location of each primer is shown in Figures 2.5 or 2.6.

| Gene Name	Locus ID	SALK	Kanamycin	Primers
SR30	At1g09140	116746	Resistant	**Fwd.1:** 5'-GCCGATCTTGATCAACTGGGC -3' **Rev.1:** 5'-CACTTGCGAATATCTCCAGGC -3'
SR34	At1g02840	106067	Resistant	**Fwd.1:** 5'-GACACAGAGTTTCGAAATGCG-3' **Rev.1:** 5'-GTTTCCCATACCTCTTAGACG-3' **Fwd.2:** 5'-GAAGGTTCCTCCAAGGCC-3' **Rev.2:** 5'-GAGATCTTGATCTTGAACGCG-3'
SR34a	At3g49430	072651	Resistant	**Fwd.1:** 5'-TTGGATGGCTGTCGCTTGAGG-3' **Rev.1:** 5'-ACTGTCTCGAGTCACCTCAGC-3'
SR34b	At4g02430	055412	Resistant	**Fwd.1:** 5'-CATCTGCGTCCTGGCAAGACC-3' **Rev.1:** 5'-CGAAACTCTGTGTCATCGAGC-3'
RSZ21	At1g23860	100950	Sensitive	**Fwd.1:** 5'-AGTGCTATGAGTGTGGTGAGC-3' **Rev.1:** 5'-ATTGGCATATGGCGAACGTCG-3'
RSZ22a	At2g24590	023090	Resistant	**Fwd.1:** 5'-ATCTCCTCCTCCTCCAAGACG-3' **Rev.1:** 5'-AACGGCAGATACAACTATGGC-3' **Rev.2:** 5'-GCAAGCATATCCAATCCACC-3'
SC35	At5g64200	033824	Resistant	**Fwd.1:** 5'-AGCAGCAGCTCCGATTGG-3' **Rev.1:** 5'-GGACCTTCGATCTCTGGG-3' **Fwd.2:** 5'-ATGTCGCACTTCGGAAGG-3' **Rev.2:** 5'-GGAACGACTGTGACTGCG-3'
SCL30	At3g55460	012838	Resistant	**Fwd.1:** 5'-CAGAAGCTGTAGAATGTAGGC-3' **Rev.1:** 5'-CATGAGAACGATCCCTGGAGC-3'
SCL30a	At3g13570	041849	Resistant	**Fwd.1:** 5'-TCTCTTGGTTCGCAACTTACG-3' **Rev.1:** 5'-CCTAAAGTGACTCGAAGAGGG-3' **Rev.2:** 5'-GCCATTATGGGGTGGTGAGCG-3'
SCL33	At1g55310	058566	Sensitive	**Fwd.1:** 5'-TCGGGATAGAAGACGTACTCC-3' **Rev.1:** 5'-ATCACTGGCTTGGTGAACGG-3' **Rev.2:** 5'-TATGCTTCTTCTAGGGCTGG-3'
RS2Z33	At2g37340	083782	Sensitive	**Fwd.1:** 5'-CAAAGATCATAGACGGGAGCC-3' **Rev.1:** 5'-TGATGGTAGGAGCAGCTACGG-3' **Fwd.2:** 5'-TAGGACCCGAGACCTTGAACG-3' **Rev.2:** 5'-TGTCCTCTCTCTCCACAACGG-3'
RS31	At3g61860	021332	Resistant	**Fwd.1:** 5'-CTCTCATATTCACCAGCC-3' **Rev.1:** 5'-ATACACAGGACTAAGAGACC-3' **Fwd.2:** 5'-ATGAGGCCAGTGTTCGTCGG-3'
RS41	At5g52040	063076	Resistant	**Fwd.1:** 5'-CTTGGAGAGGCACTTTGAGCC-3' **Rev.1:** 5'-TCCCTTCTCTTCTCATATGGC-3'
SR45	At1g16610	018237	Resistant	**Fwd.1:** 5'-GCCACGTATCATCTTCGG-3' **Rev.1:** 5'-GGAAGGTGAAGATGCTCC-3'
		004132	Sensitive	**Fwd.2:** 5'-AACGTTCACACTACCACCTCG-3' **Rev.2:** 5'-GTAAGAAGATGACCTCCCACG-3'
		123442	Resistant	**Fwd.3:** 5'-TCCACCTCCAAGGAGACTACG-3' **Rev.3:** 5'-TTAAGTTTTACGAGGTGGAGGTGG-3'
		132850	Sensitive	**Fwd.1:** 5'-GCCACGTATCATCTTCGG-3' **Rev.1:** 5'-GGAAGGTGAAGATGCTCC-3'
		121702	Resistant	**Fwd.1:** 5'-GCCACGTATCATCTTCGG-3' **Rev.1:** 5'-GGAAGGTGAAGATGCTCC-3'
T-DNA segment				**LBc:** 5'-CAAACAGGATTTTCGCCTGCTGGGG-3' **LBd:** 5'-CCCTGATAGACGGTTTTTCGCCC-3'
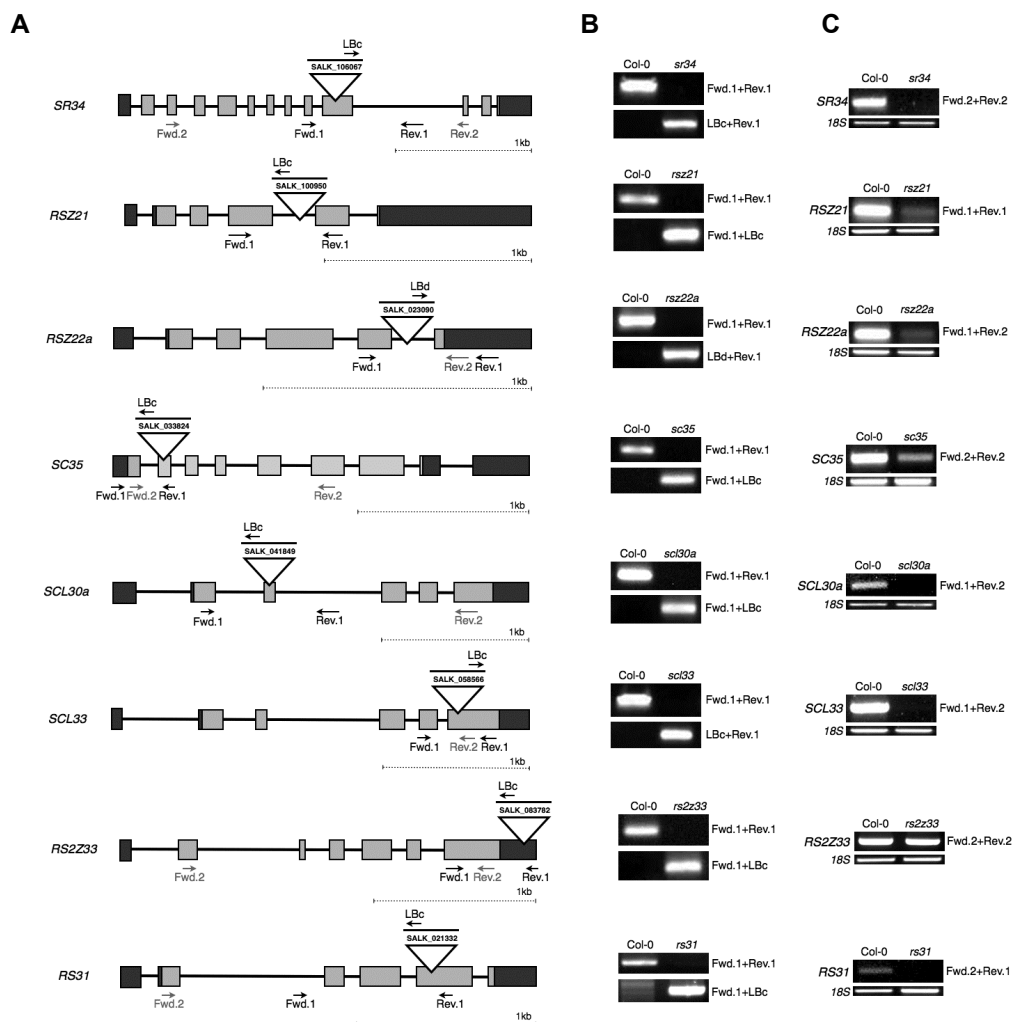
Figure 2.5. Isolation of eight loss-of-function SR protein mutants.

A. Schematic diagrams of the Arabidopsis *SR34* (At1g02840), *RSZ21* (At1g23860), *RSZ22a* (At2g24590), *SC35* (At5g64200), *SCL30a* (At3g13570), *SLC33* (At1g55310), *RS2Z33* (At2g37340) and *RS31* (At3g61860) genes showing the location of the T-DNA insertion (inverted open triangles) in each mutant line. Rectangles represent exons (darker rectangles are exons in the UTRs) and lines represent introns. **B.** Verification of homozygous insertion lines by PCR-based genotyping using primers flanking the insertion and the T-DNA-specific primers LBc or LBd (annealing specifically at the T-DNA left border). The location of each primer relative to the T-DNA insertion is indicated in A. **C.** RT-PCR analysis of the transcript levels of each gene in wild-type (Col-0) and T-DNA insertion mutant seedlings using gene-specific primers. The location of each primer is indicated in A. The *18S* gene was used as a loading control. The sequences of the primers used are shown in Tables 2.3 or 2.4.

Finally, to examine the effect of the T-DNA insertions on the expression of the disrupted SR genes, RT-PCR using gene-specific primers annealing in exonic sequences was performed on all homozygous SR mutant lines. RT-PCR analyses of the *sr34*, *sc35*, *scl30a*, *scl33* and *rs31* mutants, which contain the T-DNA insertion in exonic regions, did not allow transcript detection, except for *sc35* which showed reduced transcript levels when compared with the Col-0 wild type (Figures 2.5A and 2.5C, Table 2.4). For the *rsz21* and *rsz22a* mutant lines, in which the T-DNA insertion is located in intronic regions, transcript production was also downregulated when compared with the wild type (Figures 2.5A and 2.5C). Finally, in the *rs2z33* mutant, containing the T-DNA insertion in the 3'UTR, RNA levels were unaffected (Figures 2.5A and 2.5C). Thus, of the eight homozygous insertion lines isolated for different Arabidopsis SR genes, four appear to be true genetic null mutants.

Regarding the *SR45* gene (At1g16610), three homozygous mutant alleles were isolated. Initially, five different SALK lines were screened: SALK_018237, SALK_004132, SALK_123443, SALK_132850 and SALK_121702 (Table 2.4). However, the site of the T-DNA insertion for SALK_132850 and SALK_121702 was found to be the exact same one as for SALK_018237. Therefore, Figure 2.6 depicts only SALK_018237, SALK_123443 and SALK_004132, the three homozygous mutant lines isolated for *SR45*. The latter insertion mutant line was named *sr45-1*, in accordance to Ali et al. (2007)

For these three homozygous SR45 mutant lines, genomic PCR using primers flanking the insertions (Table 2.4) amplified the expected gene size product for the Col-0 wild type but not for any of the *SR45* mutant alleles (Figures 2.6A and 2.6B), indicating gene disruption in all lines. By contrast, PCR using the LBc or LBd primers (Table 2.4) amplified a product in the mutant lines but not in Col-0, confirming the presence of the insertion in each mutant (Figures 2.6A and 2.6B). Again, the resulting LB-specific fragments were then sequenced to verify the exact insertion location.

To examine the effect of the T-DNA insertions on the expression of the disrupted *SR45* gene, RT-PCR using gene-specific primers annealing in exonic sequences was performed. RT-PCR analysis (Table 2.4, Figure 2.6A) of the *sr45-1* mutant, which

contains the T-DNA insertion in an exonic region, did not allow detection of the *SR45* transcript (Figure 2.6C). By contrast, the SALK_123442 line, in which the T-DNA insertion is located in an intronic region, transcript production was slightly increased when compared with the wild type, while in SALK_018237, containing the T-DNA insertion in the 5'UTR, RNA levels were unaffected (Figure 2.6A and 2.6C). Hence, of the three *SR45* homozygous lines isolated, only *sr45-1* turned out to represent a loss-of-function mutant.
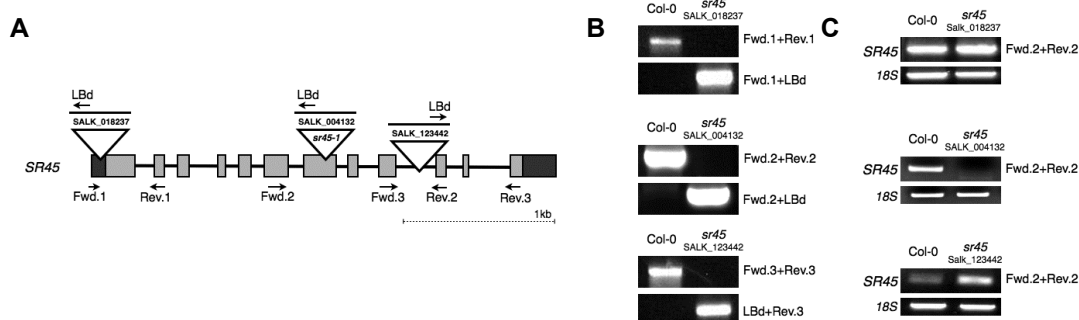

Figure 2.6. Isolation of an *SR45* loss-of-function mutant.
A. Schematic diagram of the Arabidopsis *SR45* gene (At1g16610) showing the position of the T-DNA insertion (inverted open triangles) in each mutant line. Rectangles represent exons (darker rectangles are exons in the UTRs) and lines represent introns. **B.** Verification of homozygous insertion lines by PCR-based genotyping using primers flanking the insertion and T-DNA-specific primers LBc or LBd, annealing specifically at the left border. The location of each primer relative to the T-DNA insertion is indicated in A. **C.** RT-PCR analysis of *SR45* transcript levels in Col-0 and T-DNA insertion mutant seedlings. The location of each primer is indicated in A. The *18S* gene was used as loading control. The sequences of the primers used are shown in Tables 2.3 and 2.4.

Chapters 3 and 4 of the present thesis report the physiological, biochemical and molecular characterization of the *sr45-1* mutant line (SALK_004132).

2.4. Discussion

Plants, as sessile organisms, have evolved sophisticated mechanisms that allow them to cope with adverse environmental stress. The basis of this capacity for adaptation lies ultimately at the genome level. Alternative splicing, a versatile means of regulating gene expression and generating proteomic diversity, is likely to play an essential role in plant stress responses. SR proteins, as key factors in the early recognition of splice sites and being highly conserved among genomes undergoing alternative splicing, are widely recognized as the major regulators of this key posttranscriptional regulatory mechanism. The functional analysis of plant SR-related proteins could therefore implicate these splicing factors in the response to changes in the environment, substantiating a role for alternative splicing in plant stress tolerance.

2.4.1. Tissue- and stress-specific SR gene expression

We have initiated the characterization of Arabidopsis SR genes by looking at their expression in both embryonic and vegetative tissues. Seed hydration triggers the germination process, which is accompanied by changes in gene activities. In fact, our analyses show that more than half of the Arabidopsis SR genes while being barely detected in dry seeds are clearly activated upon imbibition, suggesting that they may be important in the seed germination process. Regarding vegetative tissues, all 18 SR protein genes appear to be constitutively expressed in roots and flowers. Previous northern blot analyses of Arabidopsis SR genes (e.g. *SR34*, *RS31*, *RS40*, *RS41*, *RS2Z33*, *SR30*, *RSZ21*, *RSZ22*) also indicated expression in roots and flowers (Lazar et al., 1995; Lopato et al., 1996b; Golovkin and Reddy, 1998; Lopato et al., 1999a; Lopato et al., 2002; Kalyna et al., 2003). In particular, consistent with our results, *RS31*, *RS40* and *RS41* were reported to display highest expression in these tissues (Lopato et al., 1996b). Although *RS2Z33* expression has been reported only in roots and flowers (Lopato et al., 2002; Kalyna et al., 2003), our results indicate that this SR protein gene is also expressed in leaves, albeit at very low levels. Fang *et al.* (2004) described distinct as well as overlapping expression patterns for *SCL33, SR34* and

SR30, with the first two being more expressed in leaves than the latter. *RSZ21* and *RSZ22* expression has been reported in all tissues, but at lower levels in leaves and higher in flowers (Golovkin and Reddy, 1998), which is in agreement with our results. Consistent with the expression of *SR30* in roots, stems, leaves and flowers, overexpression of this SR splicing regulator led to severe developmental abnormalities in both embryos and young plants (Lopato et al., 1999a) reflecting the essential role of this protein in Arabidopsis. Interestingly, despite the restricted expression pattern of *RS2Z33* mainly in roots and flowers, its overexpression causes dramatic changes in overall plant development, including an increase of cell size in various tissues, abnormal shape of root hairs and trichomes, and impaired growth of pollen tubes (Kalyna et al., 2003).

Recently, a comprehensive RT-PCR analysis of the 18 Arabidopsis SR genes has shown that all are expressed in roots, stems, leaves, inflorescences and pollen, although some genes display very low transcript levels in the latter tissue (Palusa et al., 2007). This same study reported no dramatic stress-dependent changes in overall transcript levels for any SR gene except perhaps for *SCL33*, which is repressed by salt and temperature stress as well as by exogenous application of the stress phytohormone ABA. Our results indicate that this gene is also repressed by cold stress but induced by ABA. Moreover, our analyses show that the vast majority of Arabidopsis SR genes are affected by both cold and ABA treatments, which either activate or repress gene expression. These conflicting results could be linked to the conditions under which the plant material was grown and/or the ABA concentrations used or the time of exposure to cold. In fact, in our study seedlings grown in full strength MS media were treated with 30 μM ABA or exposed to 4ºC for 32 hours, whereas in the mentioned report seedlings were grown in 0.5xMS medium and treated with 20 μM ABA or exposed to 4ºC for only 24 hours.

Publicly available microarray data from Genevestigator (https://www.genevestigator.com/gv) and the Bio-Array Resource for Plant Biology (BAR) (http://esc4037-shemp.csb.utoronto.ca/welcome.htm) websites also indicate

ubiquitous expression of all Arabidopsis SR genes among embryonic and vegetative tissues. In these databases, ABA is the only hormone for which there are indications of inducing alterations in SR gene expression. More specifically, the expression of *RSZ21*, *SC35*, *SCL28*, *SCL30, SCL33*, *RS2Z32* and *RS2Z33* genes appears to be upregulated by the phytohormone. In agreement, our analysis, except for *RS2Z22*, which is downregulated, we observed that most SR genes are upregulated by ABA. Furthermore, the information available in these databases indicates that temperature stress has a great influence on SR gene expression. Indeed we have found that, except for *RSZ22, SC35* and *SCL20*, SR genes are in general either activated or repressed by cold treatment. Taken together, our results suggest an involvement of SR genes in plant stress responses to cold and ABA.

2.4.2. Alternative splicing of SR genes

The pre-mRNAs encoded by SR protein genes are themselves alternatively spliced, with this splicing being under tight spatial, temporal and developmental control (Lopato et al., 1999a; Kalyna et al., 2003; Isshiki et al., 2006; Kalyna et al., 2006; Ali et al., 2007; Palusa et al., 2007; Tanabe et al., 2007). Palusa et al. (2007) have reported that about 80% of the Arabidopsis SR genes undergo alternative splicing, producing as many as nine splice variants from a single gene. More recently, analysis of the Arabidopsis SR gene family by RNA-sequencing (RNA-seq) detected a total of 133 splice junctions, comprising 122 previously annotated and 13 novel introns (Filichkin et al., 2010). Our analysis of the alternative splicing of pre-mRNAs of all 18 SR genes in different organs and in response to ABA and cold treatments reveals alternative splicing events for only four genes — *SR30*, *SR34*, *SR2Z32* and *SR31*. It should be noted that the gene-specific primers used in this study do not allow detection of all the reported splice variants for eight of the Arabidopsis SR genes. In other cases, the variants are too close in size to be distinguished in the 1% agarose gels we used. On the other hand, differences between splice variants can be as subtle as four base pairs, which is the case of the eighth splice variant of *SR34* described in the TAIR website and by Filichkin *et al.* (2010), and therefore more readily detectable by RNA-

sequencing. Finally, many alternative splicing events occur only at very specific times in development or under certain physiological conditions, which could explain why for some SR genes many alternative splicing events remained undetected in our study.

Our analysis of the sequences of the alternatively-spliced transcripts of *SR30*, *SR34, SR2Z32* and *SR31* reveals that each contains a PTC. This is also supported by Palusa *et al.* (2007), who have described that among the 95 alternatively-spliced transcripts of SR genes about 60 harbor a PTC. It has been recently shown that half of these PTC-containing transcripts are targeted for degradation by NMD, which could provide a way of fine-tuning the abundance of functional transcripts, and thereby the abundance of SR proteins (Palusa and Reddy, 2010). Indeed, in mammalian cells, the coupling of alternative splicing and NMD provides an auto-regulatory mechanism affecting expression of SR and other splicing-related proteins (Lareau et al., 2007). The alternative transcripts observed here for *SR30*, *RS31* and *RS2Z32* are among the transcripts identified by Palusa and Reddy (2010) to constitute NMD targets. By contrast, the alternative transcript identified for *SR34* is not a target for NMD but encodes a truncated protein not very different from the full-length form except that it lacks an 18 amino acid PSK domain (Palusa and Reddy, 2010). Such a truncated protein may have altered function, different subcellular localization or be non-functional but still interact with specific spliceosomal proteins and act as a dominant negative regulator.

It has been previously reported that temperature stress strongly alters the alternative splicing of most SR genes, whereas hormones only affect this process in three SR genes — *SR34*, *SR34b* and *SCL33* (Palusa et al., 2007). Filichkin et al. (2010) have also found the full-length isoform of *SR30* to increase under light and heat stresses but decrease under cold stress, whereas the unproductive truncated form also decreases in response to cold but remains unaltered by light stress and is downregulated under heat stress. In the present study, both cold stress and exogenous application of ABA were found to affect the splicing pattern of Arabidopsis SR genes. Under cold stress, the shortest *SR30* transcript, encoding the full-length protein, is downregulated while the unproductive form remains unaltered. On the other

hand, the longest splice variant of *SR34*, which encodes a truncated protein, is no longer detectable upon cold treatment. Furthermore, the splice variants of *RS2Z32* and *RS31* encoding truncated proteins are only detectable after ABA application. These results clearly show regulation of the alternative splicing of Arabidopsis SR genes by low temperatures as well as by the phytohormone ABA, suggesting a physiological role for this posttranscriptional regulatory mechanism in plant responses to these forms of abiotic stress.

2.4.3. Expression and splicing patterns of the *SR45* gene

Our results indicate that the *SR45* gene is ubiquitously expressed in Arabidopsis. This is in accordance with previous studies reporting this gene to be present in roots, stems, leaves, influorescenses and pollen (Palusa et al., 2007; Zhang and Mount, 2009). In embryonic tissues, we found the *SR45* gene to be highly induced following seed imbibition, while in vegetative tissues expression appears to be highest in flowers and lowest in roots. Zhang and Mount (2009) reported *SR45* expression to be highest in both roots and inflorescences, while Palusa *et al.* (2007) found uniform levels of *SR45* among roots, stems and leaves, with slightly lower expression in inflorescences and pollen. Microarray expression profiles collected in Genevestigator (https://www.genevestigator.com/gv) also indicate expression of *SR45* in all plant organs, with highest levels in imbibed seeds, root tip, shoot apex, and inflorescence tissues. The differences in the expression results mentioned above are likely due to the different developmental stages at which each analysis was performed — these varied from 6-week old plants in our study, to 8-day old seedlings (Zhang and Mount, 2009) and 30-day old plants (Palusa et al., 2007).

The expression and splicing pattern of *SR45* has been reported to show no striking changes in response to different stress cues, including ABA, NaCl, heat, cold and glucose (Palusa et al., 2007). However, more recently, Zhang and Mount (2009) showed that both the relative levels of two *SR45* splice variants and of total expression of the gene do change significantly in response to sucrose and temperature stress. In particular, expression of *SR45.2* was downregulated in the absence of sucrose and in

response to cold, but upregulated in response to heat. Contrary to these results, we have observed *SR45* to be upregulated by ABA, salt and cold stresses, downregulated by heat stress, and unaffected by either glucose or drought. Again, these discrepancies could be linked to the plant's developmental stage and/or the time of exposure to the stress treatment. It should be pointed out that in addition to being expressed at very low levels when compared to *SR45.1,* the *SR45.2* splice variant is also very similar in size to SR45.1, preventing easy separation of both splice variants in standard 1% gels.

Besides the two different isoforms described for *SR45, SR45.1* and *S45.2* (Palusa et al., 2007; Zhang and Mount, 2009), a third splice variant, *SR45.3*, has been recently annotated in the TAIR database. The second isoform has been shown to be expressed at very low levels (Palusa et al., 2007; Zhang and Mount, 2009) rendering it hard to detect. Indeed, although we have been able to detect both the *SR45.1* and *SR45.2* splice variants, our results confirm that the latter is much less abundant. Importantly, Zhang and Mount (2009) reported that when Arabidopsis seedlings are grown in the absence of sucrose, *SR45.2* expression is barely detectable. This may explain why we rarely observed expression of this splice variant, as all seedlings used in our study were grown in MS medium without sucrose. Regarding *SR45.3,* it has not yet been described in any published report. This splice variant arises from the selection of an alternative 5' splice site in the ninth intron. It is possible that this isoform is only expressed at particular developmental stages, in specific tissues, under certain physiological conditions or even exclusively in response to given stress cues.

2.4.4. Isolation of loss-of-function mutants

The overexpression and knockout of individual SR genes will be of crucial importance in the analysis of the function(s) of plant SR proteins. Overexpression of two SR protein genes, At*SR30* and At*RS2Z33* was reported to lead to severe developmental abnormalities in both embryos and young plants (Lopato et al., 1999a; Kalyna et al., 2003), suggesting an essential role for these splicing factors during normal development in Arabidopsis. Regarding loss-of-function mutants, none have

yet been reported for any of the 18 Arabidopsis SR protein genes. In this study, we isolated homozygous lines for insertions in eight Arabidopsis SR genes — *SR34, RSZ21, RSZ21, RSZ22a, SC35, SCL30a, SCL33, RS2Z33* and *RS31*. Of these eight, only one is unaffected in transcript production by the T-DNA insertion (*rs2z33*), while three are knockdown mutants (*rsz21, rsz22a* and *sc35*). The remaining four, *sr34, scl30a, scl33* and *rs31,* appear to be true null mutants and therefore hold much promise for the future identification of the biological role(s) of plant SR proteins.

Finally, we isolated three homozygous lines for T-DNA insertions in the non-canonical SR protein gene *SR45*, but only one turned out to be a loss-of-function mutant. This *SR45* knockout has been previously described and named *sr45-1* (Ali et al., 2007). Reddy and coworkers described pleiotropic phenotypes for *sr45-1*, including late flowering, altered leaf and flower morphology, delayed root growth, and smaller overall plant size when compared to the wild type (Ali et al., 2007), implicating *SR45* in several aspects of plant development. However, the response of this mutant to environmental cues has not yet been addressed. The following chapters of this thesis describe the further characterization of *SR45,* identifying additional roles for this plant-specific splicing factor, specifically in sugar signaling and the response to stress.

2.5. Materials and Methods

2.5.1. Plant materials and growth conditions

Seeds from Arabidopsis (*Arabidopsis thaliana*), ecotype Col-0, were surface-sterilized and sown in petri dishes containing Murashige and Skoog (MS) salts (Duchefa Biochemie), 2.5 mM MES (pH 5.7), 0.5 mM myoinositol and 0.8% agar. Seeds were then stratified for three days at 4°C in the dark (to break dormancy) before being placed in a growth chamber under 16-h photoperiod (80 μmol.m^{-2}.s^{-1} white light) at 22°C and 60% relative humidity. After two-three weeks, plants were transferred to soil in individual pots.

For ABA, salt (NaCl) and glucose treatments, two-week old plants were transferred to new plates containing 30 μM ABA, 250 mM NaCl or 10% glucose for 2h, 48h and

10h, respectively, before harvesting of the plant material. For cold and heat stresses, two-week old plants were placed at 4ºC and 38ºC for 32h and 2h, respectively, before collection of the seedlings. For drought stress, three-week old seedlings were transferred to 3MM paper and plant material collected 4h later.

2.5.2. T-DNA insertion lines

T-DNA insertion mutations in genes encoding 13 individual SR proteins and the SR45 splicing factor (Table 2.4) were identified from a library of Arabidopsis sequence-indexed T-DNA insertion mutants (http://signal.salk.edu). The insertion lines were first selected for kanamycin resistance (Table 2.4) by plating seeds on MS medium supplemented with 50 μg/mL of kanamycin. As a precaution, in case of silencing of kanamycin resistance, some seeds were also plated on MS medium alone. For kanamycin resistant lines, only the seedlings that grew on the antibiotic were selected and transferred to soil.

The progeny of the mutant lines was genotyped by PCR using primers specific for the T-DNA vector and the gene(s) in question (Table 2.4) in order to screen for plants homozygous for the insertions. All PCR products were separated and visualized on 1% agarose gels. To confirm the exact insertion location, PCR products from each homozygous mutant were sequenced (AB DNA Sequencer 3130xl).

2.5.3. RNA analysis

Total RNA was extracted using TriReagent (SIGMA), according to the manufacturer's protocol. Reverse transcription was performed on 1 μg of total RNA using M-MLV reverse transcriptase (Promega) as instructed by the manufacturer.

In the analysis of tissue-specific expression and splicing patterns, plant material was harvested from different tissues (roots, leaves and flowers) 30 days after transfer to soil. RNA from dry and imbibed seeds was extracted as previously described (Voinnet et al., 2003). For stress-specific expression and splicing patterns, two-week old seedlings were subjected to different stress treatments before RNA extraction.

Primers were designed for each individual gene in order to detect alternatively-spliced variants described in the TAIR database (http://www.arabidopsis.org). To test the specificity of the primers, PCRs were first performed with Arabidopsis genomic DNA. The location of the primers used as well as the expected band sizes are indicated in Table 2.1. Preliminary PCRs were carried out with different cycles to determine the linear range of amplification. Based on these analyses, 25 cycles were performed for all genes except for the housekeeping *18S*, which was detected after 22 cycles.

For RNA analysis of homozygous mutants, total RNA was extracted from two-week-old seedlings. Primer pairs flanking the T-DNA insertion (Table 2.4) were designed for the *SR45* and each individual SR protein gene. For PCR amplification, 35 cycles were performed except for the internal control *18S* (Table 2.3), which was detected after 22 cycles. PCR products were separated on 1% agarose gels except for the separation of the *SR45* splice variants, where 3.5% agarose gels were used instead.

R. Carvalho was responsible for all the experimental work and data analysis.

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CHAPTER 3

The plant-specific SR45 protein negatively regulates glucose and ABA signaling during early seedling development in Arabidopsis

3.1. Abstract

The plant-specific SR45 belongs to the highly conserved family of serine/arginine-rich (SR) proteins, which play key roles in pre-mRNA splicing and other aspects of RNA metabolism. An *Arabidopsis thaliana* loss-of-function mutant, *sr45-1*, displays pleiotropic phenotypes, such as defects in flower and leaf morphology, root growth and flowering time. Here we show that the *sr45-1* mutation confers hypersensitivity to glucose during early seedling growth in Arabidopsis. Unlike wild-type plants, the *sr45-1* mutant displays impaired cotyledon greening and expansion as well as reduced hypocotyl elongation of dark grown seedlings when grown in the presence of low (3%) glucose concentrations. In addition, SR45 is involved in the control of glucose-responsive gene expression, as the mutant displays enhanced repression of photosynthetic and nitrogen metabolism genes and overinduction of starch and anthocyanin biosynthesis genes. Like many other sugar-response mutants, *sr45-1* also shows hypersensitivity to abscisic acid (ABA), but appears to be unaffected in ethylene signaling. Importantly, the *sr45-1* mutant shows enhanced ability to accumulate ABA in response to glucose, and the ABA biosynthesis inhibitor fluridone partially rescues the sugar-mediated growth arrest. Moreover, three ABA biosynthesis genes and two key ABA signaling genes, *ABI3* and *ABI5*, are markedly overinduced by glucose in *sr45-1*. These results provide evidence that the SR45 protein defines a novel player in plant sugar response that negatively regulates glucose signaling during early seedling development by downregulating both glucose-specific ABA accumulation and ABA biosynthesis and signaling gene expression.

3.2. Introduction

To adapt to environmental and metabolic changes, higher plants can sense sugar levels and adjust growth and development accordingly, modulating various important processes including germination, early seedling development, leaf and root morphogenesis, flowering, defense responses and senescence, as well as gene

expression (Koch, 1996; Gibson, 2005; Rolland et al., 2006). While sucrose is the major sugar translocated in plants, glucose plays a preponderant regulatory role, being recognized as a central signaling molecule in addition to a universal carbon and energy source. The first enzyme in glycolysis, hexokinase (HXK), which phosphorylates glucose to glucose-6-phosphate, is the evolutionarily conserved glucose sensor in a wide range of organisms, from yeast to mammals, including plants (Rolland et al., 2006), where both HXK-dependent and HXK-independent glucose signal transduction pathways appear to coexist (Xiao et al., 2000).

In *Arabidopsis thaliana*, cotyledon greening and expansion as well as initiation of true leaf development are suppressed during growth in the presence of 6% glucose, with the majority of wild-type seedlings exhibiting a dramatic developmental arrest (Jang et al., 1997). This severe inhibition of early seedling growth by elevated sugar levels has been broadly used to monitor the response to sugars and screen for sugar signaling mutants. Characterization of these mutants has revealed that several of them are also impaired in phytohormone metabolism or response, disclosing extensive interactions between glucose and hormone signaling pathways that modulate plant growth and metabolism.

Among the plant hormones, abscisic acid (ABA) and ethylene are of major importance in the interaction with sugar signals. ABA mediates a developmental checkpoint that arrests early growth under adverse environmental conditions (Lopez-Molina et al., 2001), and high levels of exogenous glucose similarly repress cotyledon development and hypocotyl elongation. Glucose has also been shown to induce the expression of ABA synthesis and signaling genes and to increase endogenous ABA levels, while the ABA-deficient *aba2* mutant fails to arrest in 6% glucose (Cheng et al., 2002). These data suggest that glucose-specific ABA accumulation is needed for sugar signaling during early seedling growth. Most ABA biosynthesis and ABA insensitive mutants described so far are insensitive to high glucose concentrations. Conversely, ethylene insensitive mutants such as *etr1*, *ein2* and *ein3* are glucose hypersensitive, whereas the ethylene overproduction mutant *eto1* and the constitutive ethylene triple response mutant *ctr1* display glucose insensitivity. Furthermore, the

ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) prevents inhibition of cotyledon greening and expansion at high concentrations of glucose (Zhou et al., 1998). Ethylene may antagonize the glucose response partially through repression of ABA biosynthesis, as suggested by the increased ABA levels present in the *ein2* mutant (Ghassemian et al., 2000; Cheng et al., 2002).

Serine/arginine-rich (SR) proteins constitute an important class of essential splicing factors that is highly conserved in higher eukaryotes. The splicing of introns from the precursor-mRNA (pre-mRNA) is carried out by the spliceosome, which consists of five small nuclear ribonucleoproteins (snRNPs) and many additional proteins. Members of the SR protein family are non-snRNP spliceosomal factors that have been shown in animal systems to play vital roles in the most crucial steps of spliceosome assembly (Wu and Maniatis, 1993; Kohtz et al., 1994; Shen and Green, 2004). They share a multidomain structure typically characterized by the presence of one or two N-terminal RNA-Recognition Motifs (RRMs) and a C-terminal reversibly phosphorylated arginine/serine-rich (RS) domain. Binding of SR proteins to the pre-mRNA is mediated by the RRM, while the RS domain is involved in protein-protein interactions that facilitate recruitment of core splicing machinery components to nearby splice sites (Wu and Maniatis, 1993). Therefore, SR proteins influence splice site selection in a concentration-dependent manner (Graveley, 2000), being pivotal in alternative splicing. Some SR proteins that shuttle between the nucleus and the cytoplasm play additional roles in RNA metabolism, including mRNA export (Huang and Steitz, 2001), stability and quality control (Zhang and Krainer, 2004), as well as translation (Sanford et al., 2004).

Plants possess about twice as many SR proteins as animals. Owing to large interchromosomal duplications, the Arabidopsis genome encodes 19 SR proteins (Kalyna and Barta, 2004; Reddy, 2004; Barta et al., 2008), 11 of which display a unique domain organization not found in any metazoan organism (Barta et al., 2008). Functional studies of three individual Arabidopsis SR protein genes have been reported — ectopic expression of SRp30 (Lopato et al., 1999) and RSZ33 (Kalyna et al., 2003), as well as loss-of-function of the SR45 protein (Ali et al., 2007; Zhang and

Mount, 2009) — all revealing pleiotropic morphological and developmental effects, as well as altered splicing patterns of other SR protein genes.

SR45 is the sole member of a plant-specific Arabidopsis SR protein subfamily that is highly conserved in the plant kingdom and seems to have appeared later in evolution in flowering plants (Ali et al., 2007). It displays a highly atypical SR protein structure, with a single RRM flanked by two RS domains, and has been shown to function as an essential splicing factor in an in vitro heterologous complementation assay (Ali et al., 2007). Its nuclear distribution in speckles is regulated by phosphorylation and transcription (Ali et al., 2003; Ali and Reddy, 2006), and it interacts in vitro with the Arabidopsis SR33, AFC2 kinase and U1-70K (Golovkin and Reddy, 1999). The latter protein has been found to interact in vivo with both RS domains of SR45 within nuclear speckles (Ali et al., 2008). Activity of the *SR45* promoter appears to be largely confined to actively growing and dividing cells (Zhang and Mount, 2009), and the only Arabidopsis SR protein loss-of-function mutant reported so far, *sr45-1*, displays general delayed development, including late flowering and slower root growth, as well as altered leaf and flower morphology (Ali et al., 2007). One of two alternatively-spliced SR45 isoforms was found to complement exclusively the mutant's flower phenotype, while the other rescues only the root defect (Zhang and Mount, 2009), thus ascribing functional significance to alternative splicing of this Arabidopsis gene.

This study reports phenotypic and molecular analyses of the *sr45-1* mutant showing that it is defective in glucose control of cotyledon development, hypocotyl elongation in the dark and gene expression. Our results indicate that the SR45 protein defines a negative regulator of sugar signaling during early seedling development in Arabidopsis involved in the repression of glucose-induced ABA accumulation and downregulation of ABA biosynthesis and signaling gene expression.

3.3. Results

3.3.1. The *sr45-1* mutant is hypersensitive to glucose inhibition of early seedling development

An *SR45* loss-of-function mutant, *sr45-1*, has been previously reported to display pleiotropic developmental phenotypes, such as delayed flowering and root growth, as well as changes in leaf and flower morphology (Ali et al., 2007). In the present study, seed germination and early seedling development of the same T-DNA insertion mutant (SALK_004132) were evaluated.

When *sr45-1* mutant seeds were plated on MS medium under standard growth conditions, radicle emergence, cotyledon greening and cotyledon expansion occurred slightly later than with Col-0 wild-type seeds — about two additional days were necessary for the mutant to attain full germination and cotyledon development rates, but no differences between mutant and wild-type seedlings were apparent by day 4 after stratification (data not shown). This slight delay in germination and early seedling development was not significantly altered under different abiotic stress conditions, such as high salinity, drought, cold or heat stress (data not shown).

As seen in Figure 3.1A, when grown on control media for 7 days, Col-0 and *sr45-1* seedlings behaved similarly, reaching full seed germination (radicle emergence) and cotyledon development rates. However, although the exogenous supply of 3% glucose had no effect on Col-0 plants nor on radicle emergence of *sr45-1* seeds, both the greening and expansion of mutant cotyledons were severely affected by the presence of the sugar, with *sr45-1* seedlings only being able to green about 5% and expand around 40% of their cotyledons. In contrast to glucose, the effect of mannitol was similar in wild type and mutant for the three parameters (Figure 3.1A). Thus, in the presence of glucose concentrations as low as 3%, unlike Col-0 seedlings, *sr45-1* exhibited an arrest in seedling development, displaying small, purple cotyledons that show little expansion (Figure 3.1B). Very similar results were obtained in the presence of equimolar concentrations of sucrose (data not shown). However, the developmental

difference between wild-type and mutant plants was not observed when both genotypes were grown in the presence of equimolar concentrations of mannitol (Figure 3.1B), indicating that the growth arrest is sugar-specific and not solely due to osmotic stress.
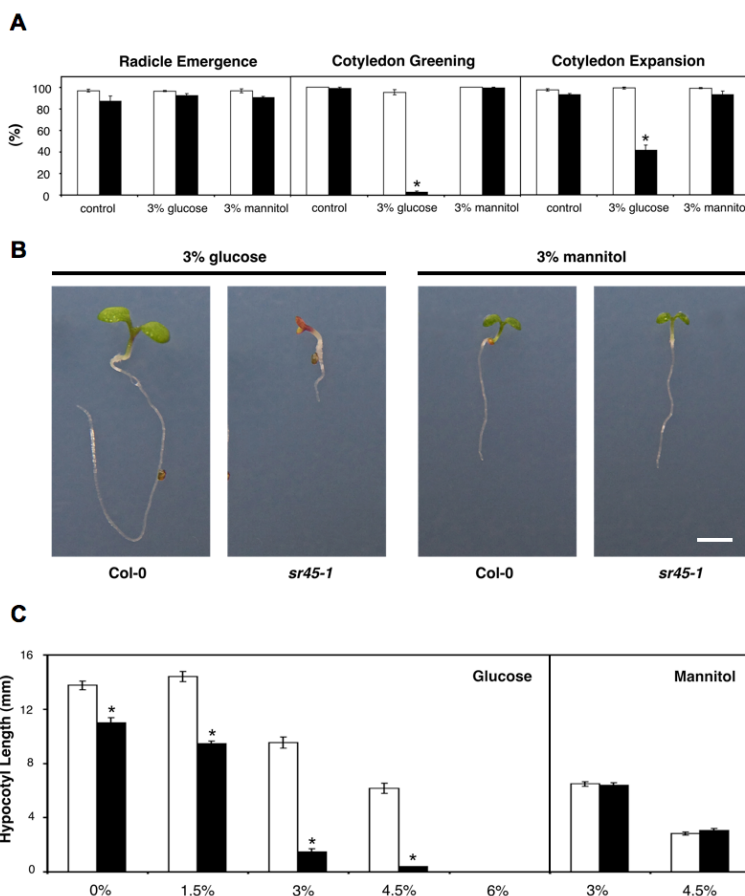

Figure 3.1. Glucose phenotypes of the *sr45-1* mutant.
A. Germination (radicle emergence) and early seedling development (cotyledon greening and expansion) rates, scored 7 days after stratification, of Col-0 (open bars) and *sr45-1* (closed bars) seedlings grown in control conditions or in the presence of 3% glucose or 3% mannitol (means ± SE, *n*=3). **B.** Representative images of wild-type (Col-0) and mutant (*sr45-1*) seedlings grown in the presence of 3% glucose or 3% mannitol for 7 days (scale bar, 2 mm). **C.** Hypocotyl length, measured 6 days after stratification, of Col-0 (open bars) and *sr45-1* (closed bars) seedlings grown in the dark under different glucose or mannitol concentrations (means ± SE, *n*=30-50). Asterisks indicate significantly different values (*P* < 0.01) from the corresponding wild type according to Student's *t*-test.

The *sr45-1* mutation also increases the sensitivity to the glucose inhibitory effects on hypocotyl elongation of dark-grown Arabidopsis seedlings. Consistent with a general delay in development (Ali et al., 2007), *sr45-1* seedlings displayed slightly shorter hypocotyls under control conditions, but the glucose-dependent reduction in hypocotyl elongation was strongly enhanced in *sr45-1* seedlings, with mutant hypocotyl length being about 20% of that of wild type in the presence of 3% glucose (Figure 3.1C). The *sr45-1* mutant exhibited similar hypocotyl length to the wild type in the presence of 3% and 4.5% mannitol (Figure 3.1C), again showing that the mutation specifically affects responses to glucose but not to the osmotic stress it induces.

3.3.2. Expression of the *SR45* gene in the *sr45-1* mutant background rescues the glucose phenotype

To ensure that the *sr45-1* glucose phenotypes are a consequence of a single gene mutation in *SR45*, the *sr45-1* mutant was transformed with the wild-type gene. The coding sequences of both isoform 1 (*SR45.1*), the longest and most expressed splice variant of *SR45*, and isoform 2 (*SR45.2*), which could barely be detected in young Arabidopsis seedlings, were cloned under the control of the 35*S* promoter and infiltrated into mutant plants. Three independent lines expressing either *SR45.1* or *SR45.2* in the *sr45-1* mutant background (Figure 3.2A) were isolated and characterized. Importantly, both the slight delay in early development observed under control conditions (data not shown) and the inhibition of early plant growth in the presence of 3% glucose was reverted in all six transgenic lines (Figure 3.2B). Both *SR45.1* and *SR45.2* complementation lines showed complete restoration of wild type cotyledon greening (Figure 3.2C). Also regarding the glucose-dependent shortening of the hypocotyl, the three *SR45.2* and two *SR45.1* (C1 and C3) complementation lines allowed total phenotype restoration, while line C2 displayed ~70% rescue of the hypocotyl elongation phenotype (Figure 3.2D).
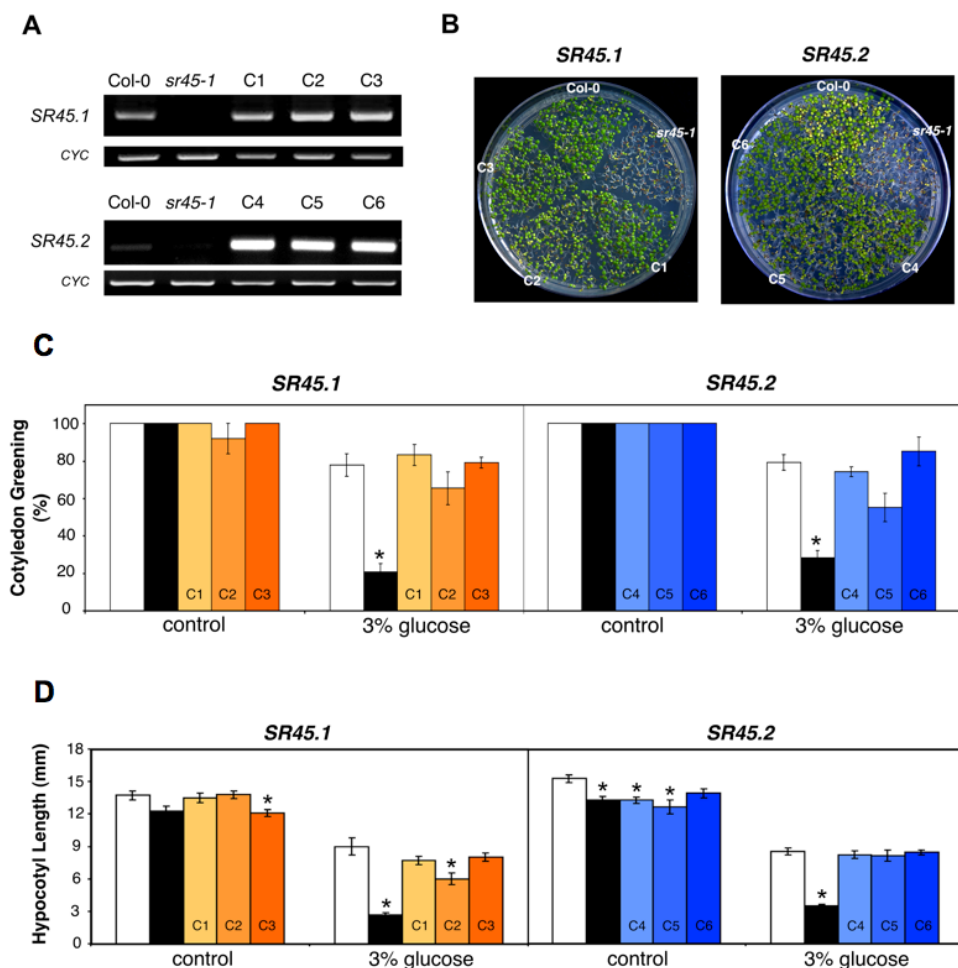
Figure 3.2. Complementation of the *sr45-1* mutant glucose phenotype.

A. RT-PCR analysis of the transcript levels of two *SR45* isoforms (*SR45.1* and *SR45.2*) in seedlings of wild type (Col-0), mutant (*sr45-1*) and six independent complementation lines carrying either the *SR45.1* (C1, C2, C3) or the *SR45.2* (C4, C5, C6) wild-type coding sequence under the control of the 35S promoter (*cyclophilin* was used as a loading control). **B.** Representative images of Col-0, *sr45-1* and C1, C2 and C3 or C4, C5 and C6 seedlings grown in the presence of 3% glucose for 7 days. **C.** Cotyledon greening rates, scored 7 days after stratification, of Col-0 (open bars), *sr45-1* (closed bars) and C1, C2 and C3 (orange bars) or C4, C5 and C6 (blue bars) seedlings grown in control conditions or in the presence of 3% glucose (means ± SE, *n*=3). **D.** Hypocotyl length, measured 6 days after stratification, of Col-0 (open bars), *sr45-1* (closed bars) and C1, C2 and C3 (orange bars) or C4, C5 and C6 (blue bars) seedlings grown in the dark in control conditions or in the presence of 3% glucose (means ± SE, *n*=20-40). Asterisks indicate significantly different values ($P < 0.01$) from the corresponding wild type according to Student's *t*-test.

3.3.3. Glucose-responsive gene expression is altered in the *sr45-1* mutant

In addition to modulating a number of developmental processes, sugars are well known to regulate transcriptionally a wide variety of genes, including those involved in photosynthesis, carbohydrate, nitrogen and secondary metabolism. Elevated sugar levels have long been known to downregulate the transcription of both photosynthetic genes and genes associated with nitrogen metabolism, while upregulating the expression of genes involved in the synthesis of polysaccharides and pigments (Koch, 1996). In order to investigate whether the *sr45-1* mutant also displays a glucose hypersensitivity phenotype at the molecular level, expression of several genes regulated by the glucose signaling pathway was analyzed by RT-PCR in wild-type and mutant seedlings grown in control conditions or in the presence of 3% glucose or 3% mannitol.

Figure 3.3 shows that although in Col-0 seedlings 3% glucose was insufficient to repress two photosynthetic genes — *RBCS*, encoding the small subunit of ribulose-1,5-bisphosphate carboxylase, and *CAB1*, encoding a chlorophyll a/b binding protein — both were clearly downregulated by the same glucose concentration in *sr45-1* seedlings. Similarly, 3% glucose exerted only a slight repression on the nitrogen metabolism gene *ASN1* (*asparagine synthetase 1*) in wild-type seedlings, but strongly repressed its expression in the mutant. Furthermore, *APL3*, encoding the large subunit of ADP-glucose pyrophosphorylase (*AGPase*), a key enzyme in starch synthesis, and the anthocyanin biosynthesis gene *chalcone synthase* (*CHS*) were clearly overinduced by 3% glucose in *sr45-1* (Figure 3.3). Exposure to 3% mannitol, used as an osmotic control, did not affect appreciably the expression of any of the analyzed genes (Figure 3.3), indicating that the observed alterations in transcript levels are glucose-specific.
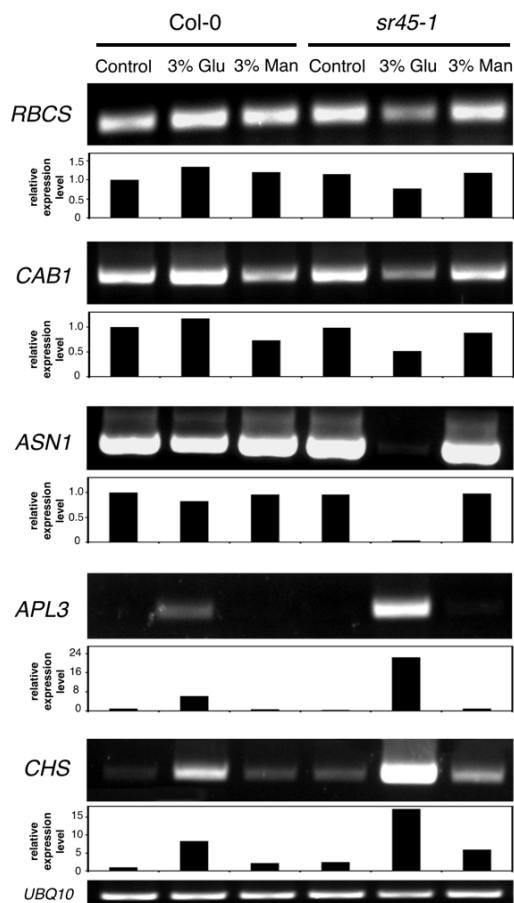
Figure 3.3. Regulation of glucose-responsive gene expression in the *sr45-1* mutant.

RT-PCR analysis of the transcript levels of *RBCS* (*ribulose-1,5-bisphosphate carboxylase*, small subunit), *CAB1* (*chlorophyll A/B binding protein 1*), two *ASN1* (*asparagine synthetase 1*) splice variants, *APL3* (*ADP-glucose pyrophosphorylase*, large subunit) and *CHS* (*chalcone synthase*) in wild-type (Col-0) and mutant (*sr45-1)* seedlings grown in control conditions or in the presence of 3% glucose (Glu) or 3% mannitol (Man). *Ubiquitin10* (*UBQ10*) was used as a loading control. Amplified bands were quantified and relative expression levels determined using *UBQ10* as a reference, with the expression of each gene in wild-type Col-0 in control conditions set to one. Results are representative of at least three independent experiments.

3.3.4. The *sr45-1* mutant is unaffected in ethylene signaling but displays ABA hypersensitivity and enhanced glucose-induced ABA accumulation.

Previous studies have demonstrated central roles for the phytohormones ethylene and ABA in plant glucose responses (Zhou et al., 1998; Arenas-Huertero et al., 2000; Ghassemian et al., 2000; Cheng et al., 2002). It was therefore of major importance to investigate the effect of the *sr45-1* mutation on ethylene and ABA signaling.

In the presence of ethylene, dark-grown seedlings undergo morphological modifications, referred to as the ethylene triple response, which include shortening of the hypocotyls. In order to determine the response of the *sr45-1* mutant to this phytohormone, hypocotyl lengths of seedlings grown in the dark in the presence of increasing concentrations of the ethylene precursor ACC and in the absence of exogenous sugar were measured. As shown in Figure 3.4A, the *sr45-1* mutation did not alter sensitivity to ACC, with hypocotyl elongation of wild-type and mutant seedlings decreasing to a similar extent with the increase in ethylene production. Ethylene is also known to antagonize the glucose response, and the developmental arrest induced by 6% glucose in Col-0 seedlings was prevented by addition of 50 μM ACC to the growth medium (Figure 3.4B), as described previously (Zhou et al., 1998). Similarly, although 6% glucose exerted a stronger effect on the development of the hypersensitive *sr45-1* mutant, addition of ACC to this high glucose concentration allowed rescue of both cotyledon greening and expansion in *sr45-1*. This rescue was also observed in 3% glucose, where the mutant's early development is affected to a similar extent as that of the wild type in 6% glucose (Figure 3.4B). Taken together, these results indicate that *sr45-1* is unaffected in ethylene signaling.
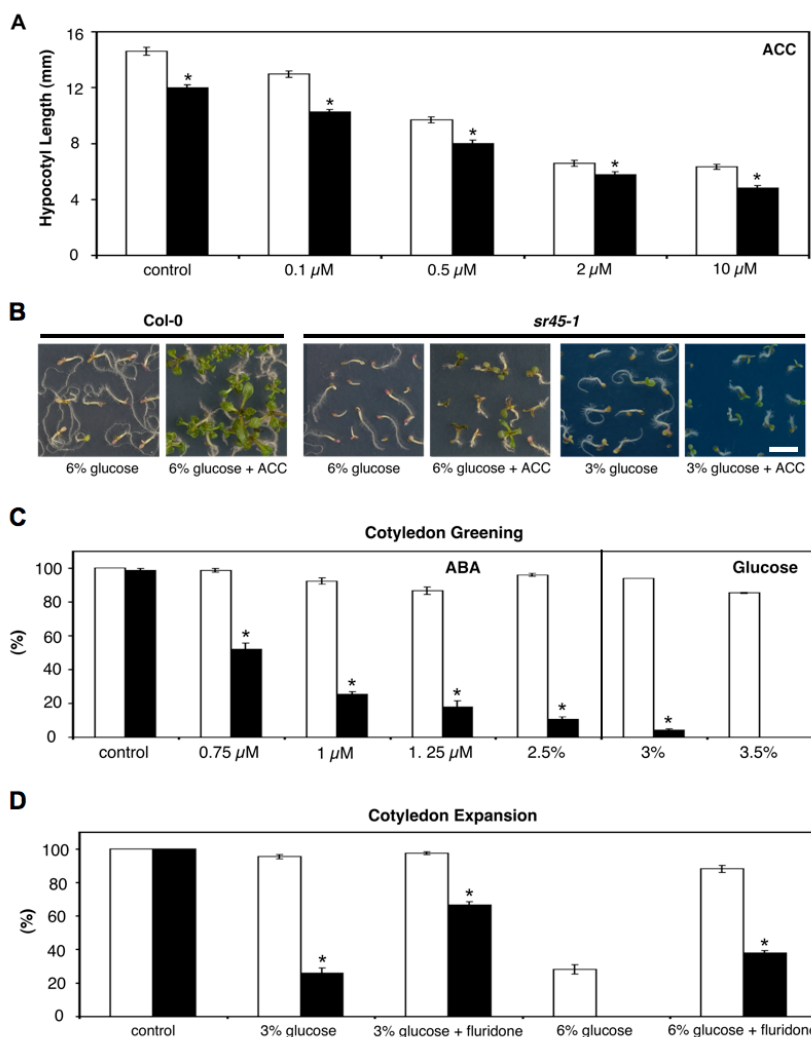
Figure 3.4. Ethylene and ABA phenotypes of the *sr45-1* mutant.
A. Hypocotyl length, measured 6 days after stratification, of Col-0 (open bars) and *sr45-1* (closed bars) seedlings grown in the dark in control conditions or in the presence of different concentrations of the ethylene precursor ACC (means ± SE, *n*=60-80). **B.** Representative images of Col-0 and *sr45-1* seedlings grown in the presence of glucose supplemented or not with 50 µM ACC for 15 days (scale bar, 3.3 mm). **C.** Comparison of cotyledon greening rates, scored 7 days after stratification, of Col-0 (open bars) and *sr45-1* (closed bars) seedlings grown in control conditions or in the presence of different concentrations of glucose or ABA (means ± SE, *n*=3). **D.** Cotyledon expansion rates, scored 7 days after stratification, of Col-0 (open bars) and *sr45*-1 (closed bars) seedlings grown in control conditions or in the presence of 3% or 6% glucose supplemented or not with 1 µM fluridone (means ± SE, *n*=3). Asterisks indicate significantly different values ($P < 0.01$) from the corresponding wild type according to Student's *t*-test.

An ABA sensitivity assay in the absence of exogenous sugar was conducted to investigate whether the glucose phenotype conferred by the *sr45-1* mutation is accompanied by altered sensitivity to ABA, as appears to be the case for the vast majority of the identified sugar response mutants. The exogenous application of ABA delayed germination of Col-0 and *sr45-1* seeds to a similar extent, with both genotypes reaching full germination rates 7 days after stratification (data not shown). However, exogenous application of ABA affected cotyledon greening to a higher extent in the *sr45-1* mutant than in the wild type (Figure 3.4C). In 0.75 µM ABA, Col-0 seedlings attained full cotyledon greening rates, whereas the mutant was only able to green about 50% of its cotyledons. Application of higher ABA concentrations (1 µM and 1.25 µM) resulted in a more pronounced decrease of cotyledon greening rates in *sr45-1* (to 20-25%) when compared with Col-0 seedlings (to ~90%). These results show that the *sr45-1* mutant is also hypersensitive to the stress hormone ABA. Nonetheless, exogenous application of glucose concentrations exerting a comparable effect to ABA on wild-type seedlings led to a more drastic arrest in the mutant's early seedling development (Figure 3.4C), indicating that the *sr45-1* mutation confers higher sensitivity to glucose than to the stress hormone.

Several lines of evidence indicate that glucose-specific ABA accumulation is required for sugar signaling during early seedling growth (Cheng et al., 2002). In agreement with this, wild-type seedlings grown in 6% glucose fail to arrest development in the presence of fluridone, an inhibitor of ABA biosynthesis (Ullah et al., 2002; Lin et al., 2007). We examined the behavior of the *sr45-1* mutant in the presence of this inhibitor to investigate whether ABA accumulation is required for the growth arrest in glucose. As fluridone blocks carotenoid synthesis, which leads to an albino-like seedling phenotype (Henson, 1984), only cotyledon expansion rates could be scored. The presence of 3% glucose had no effect on the early development of Col-0 seedlings and, as expected, the growth arrest observed in 6% glucose was reverted upon the addition of fluridone (Figure 3.4D). On the other hand, *sr45-1* seedlings expanded 25-40% of their cotyledons in the presence of 3% glucose alone (Figures 3.1A and 3.4D), but when this medium was supplemented with fluridone, cotyledon

expansion rates increased to about 65%. This partial rescue of the *sr45-1* phenotype was also seen in 6% glucose, where mutant seedlings were unable to expand their cotyledons altogether, but around 35% expansion rates were scored after supplementation with fluridone (Figure 3.4D).

The fact that treatment with fluridone partially rescued the glucose inhibition of cotyledon expansion in the mutant suggests that the growth arrest is operating, at least to some extent, via glucose-induced ABA biosynthesis. To test this hypothesis, we determined ABA endogenous levels in Col-0 and *sr45-1* seedlings grown in control media or in the presence of 3% glucose, and included the *aba2* (Leon-Kloosterziel et al., 1996) and *abi4* (Finkelstein, 1994) mutants as controls. Table 3.1 shows that there was no significant difference in ABA content between the four genotypes in the absence of sugar. As expected, the ABA-deficient mutant *aba2* was unable to respond to the presence of 3% glucose, which was also insufficient to induce a significant increase of ABA endogenous levels in both Col-0 wild-type seedlings and the ABA-insensitive *abi4* mutant. However, *sr45-1* seedlings responded to the presence of 3% glucose by increasing their ABA content nearly three-fold, suggesting that the hypersensitivity of the mutant to glucose is due to enhanced endogenous accumulation of ABA.

Table 3.1. Effect of glucose treatment on endogenous ABA levels of wild-type and mutant seedlings.

	Control	3% Glucose
Col-0	7.28 ± 1.07 [a]	10.74 ± 1.74 [a]
sr45-1	6.67 ± 1.75 [a]	18.18 ± 1.68 [b]
aba2-1	10.54 ± 1.42 [a]	9.86 ± 1.48 [a]
abi4-101	6.94 ± 0.46 [a]	8.33 ± 0.61 [a]

Quantification of ABA levels (expressed in ng ABA/g fresh weight) in Col-0 and *sr45-1* seedlings grown in control conditions or in the presence of 3% glucose (means ± SE, *n*=6). The ABA-deficient *aba2-1* mutant and the ABA-insensitive *abi4-101* mutant are shown as controls. Different letters indicate significantly different values ($p<0.05$, Student's t-test).

3.3.5. Transcription of ABA biosynthesis and signaling genes is overinduced by glucose in the *sr45-1* mutant

The enhanced ability of the *sr45-1* mutant to accumulate ABA in response to glucose prompted us to examine glucose regulation of ABA biosynthesis gene expression in wild-type and mutant seedlings. RT-PCR analysis of the five key genes in the ABA biosynthetic pathway — *ABA1*, *NCED3*, *ABA2*, *ABA3* and *AAO3* — is shown in Figure 3.5A. The *NCED3* transcript was barely detectable in the wild type, and exposure of these seedlings to 3% glucose only resulted in induced expression of *ABA1* and *ABA2*. By contrast, expression of all five genes was clearly induced by glucose in *sr45-1*, with *NCED3*, *ABA3* and *AAO3* being markedly activated in the mutant, but not at all in the wild type. This is consistent with a three-fold increase in the mutant's ABA content, indicating that the SR45 protein is involved in glucose-mediated repression of ABA biosynthesis gene expression. It has recently been reported that the glucose induced delay of rice seed germination is mediated by suppression of ABA catabolism rather than enhancement of ABA biosynthesis (Zhu et al., 2009). We therefore also analyzed the expression levels of the four Arabidopsis *CYP707A* genes, but no significant differences were observed between wild-type and mutant seedlings both under control conditions and in the presence of glucose (data not shown).

The ABA-induced postgermination arrest depends on functional *ABI3* and *ABI5* genes, with ABI5 acting downstream of ABI3 to execute this developmental checkpoint (Lopez-Molina et al., 2002). These two key ABA signaling genes encode transcription factors that are activated by both ABA and glucose (Lopez-Molina et al., 2001; Cheng et al., 2002) and appear to mediate also the glucose-induced arrest during early seedling growth (Rolland et al., 2006). We therefore investigated whether transcription of *ABI3* and *ABI5* would be misregulated in *sr45-1* seedlings, which are hypersensitive to glucose and to ABA during early seedling development.
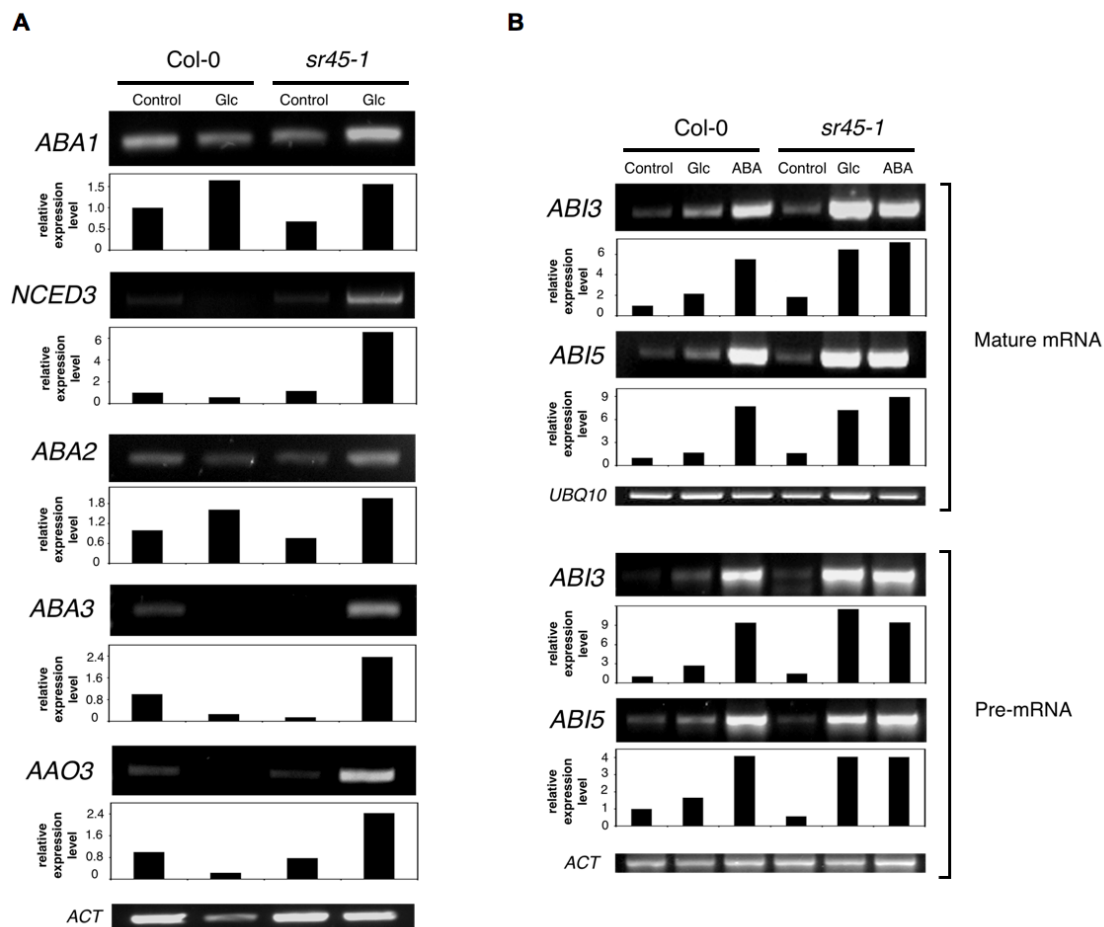
Figure 3.5. Regulation of ABA biosynthesis and signaling gene expression in the *sr45-1* mutant.
A. RT-PCR analysis of the transcript levels of ABA biosynthesis genes, *ABA1*, *NCED3*, *ABA2, ABA3* and *AAO3*, in wild-type (Col-0) and mutant (*sr45-1*) seedlings grown in control conditions or in the presence of 3% glucose (Glu). **B.** RT-PCR analysis of *ABI3* and *ABI5* transcript levels in Col-0 and *sr45-1* seedlings grown in control conditions or in the presence of 3% glucose (Glu) or 1μM ABA. To detect pre-mRNA, after reverse transcription using random primers, PCR amplification was performed using one primer annealing in an intronic sequence. *Ubiquitin10* (*UBQ10*) and *actin* (*ACT*) were used as loading controls. Amplified bands were quantified and relative expression levels determined using *UBQ10* or *ACT* as a reference, with the expression of each gene in wild-type Col-0 in control conditions set to one. Results are representative of at least three independent experiments.

As expected, RT-PCR analysis showed that *ABI3* and *ABI5* transcript levels are induced by ABA and glucose in wild-type Col-0 seedlings (Figure 3.5B). This induction is striking in the presence of 1 μM ABA, but also present at the relatively low concentration of 3% glucose. In the *sr45-1* mutant, by contrast, induction of the expression of these genes by 3% glucose was much more pronounced, with both transcripts being highly overinduced by glucose but not by ABA. This is consistent with the mutant's higher sensitivity to glucose and points to involvement of SR45 in transcriptional downregulation of *ABI3* and *ABI5* in the presence of the sugar. When pre-mRNA levels were analyzed instead of the mature *ABI3* and *ABI5* mRNA, very similar results were obtained (Figure 3.5B). This strongly suggests that the processing of these transcripts is unaffected and that the changes in steady state levels of the spliced transcripts observed in *sr45-1* reflect changes in transcription rates.

The alternative splicing pattern of other SR protein genes has been found to be altered in transgenic lines overexpressing *Arabidospis* SRp30 (Lopato et al., 1999) and RSZ33 (Kalyna et al., 2003), as well as in the *sr45-1* mutant (Ali et al., 2007). In an attempt to identify additional putative direct target transcripts of the SR45 protein, a candidate gene strategy was employed. About 25 genes predicted to undergo alternative splicing — including those coding for glucose-responsive transcription factors and ABA, ethylene or stress signaling components (Table 3.2) — were selected from the TAIR database (http://www.arabidopsis.org) and their splicing patterns analyzed by RT-PCR. Although in a few cases the predicted splice variants were not all detectable by RT-PCR at the early development stage under study (Table 3.2), comparisons between wild-type and mutant seedlings grown under control conditions or in the presence of glucose revealed no apparent changes in alternative splicing for any of the analyzed genes (data not shown).

Table 3.2. List of genes for which the splicing pattern was compared in Col-0 and *sr45-1* seedlings grown in the absence or presence of glucose

Gene name	Gene locus	# predicted splice variants	# detected splice variants
ABF3	At4g34000	3	2
AFC2 lammer kinase	At4g24740	2	2
bHLHTF	At5g65640	2	2
BMY1	At4g15210	2	2
bZIP63	At5g28770	3	3
Clathrin adaptor complexes medium subunit-related	At5g05010	2	2
CTR1	At5g03730	2	2
Dehidration responsive family	At1g26850	3	3
Dormancy/auxin associated family protein	At1g56220	3	2
DRO	At4g02200	3	2
EIN4	At3g04580	2	2
ERFTF	At1g72360	2	2
EXP2	At1g28100	7	3
FCA	At4g16280	2	2
IDH2	At2g17130	2	2
KH-NOVA	At5g04430	2	2
KIN11	At3g29160	3	2
MYBTF	At1g19000	2	2
PTB2a	At3g01150	4	2
RBCS3B	At5g38410	2	2
XBAT35	At3g23280	2	2
SEN1	At4g35770	3	2
SRM300	At1g07350	3	3
U1-70K	At3g50670	2	2
WRKYTF	At5g07100	2	2

3.4. Discussion

The *sr45-1* Arabidopsis mutant displays pleiotropic phenotypes, including late flowering, altered leaf and flower morphology, delayed root growth and smaller overall plant size when compared to the wild type (Ali et al., 2007). In the present report, we show that the *sr45-1* mutation also enhances glucose sensitivity during early seedling growth both under light and dark conditions. In the presence of exogenous glucose, the *sr45-1* mutant shows a drastic delay in the greening and expansion of cotyledons under long-day photoperiod, as well as severe inhibition of hypocotyl elongation in dark-grown seedlings. These altered glucose responses are not due to osmotic stress hypersensitivity and result from loss of function of the *SR45* gene, showing that the SR45 protein is a negative regulator of sugar signaling during early seedling development in Arabidopsis.

Alternative splicing generates two *SR45* splice variants (Palusa et al., 2007; Zhang and Mount, 2009), with *SR45.1* containing a 21-nucleotide sequence that is absent from *SR45.2*. Zhang and Mount (2009) recently showed that *SR45.1* complements the floral but not the root phenotype reported for the *sr45-1* mutant (Ali et al., 2007), while *SR45.*2 is only able to complement the defect in root growth. In the young Arabidopsis seedlings analyzed in our study, *SR45.1* was largely the most expressed splice variant, with *SR45.2* being barely detectable. However, ectopic expression of either isoform was able to complement the *sr45-1* glucose phenotypes, rescuing both the arrest in cotyledon development and the inhibition of hypocotyl elongation in the dark. These results indicate that the alternatively-spliced region of *SR45* does not play a role in glucose signaling during early seedling growth.

The *sr45-1* mutant also displays a glucose phenotype at the molecular level. Consistent with the growth arrest and hypocotyl shortening observed at low glucose concentrations, the *sr45-1* mutation confers enhanced glucose-responsive gene expression. High sugar levels typically promote the expression of genes associated with carbohydrate consumption and storage, whereas the expression of genes associated with carbohydrate production, mobilization and conservation is reduced

(Koch, 1996). In the *sr45-1* mutant, 3% glucose was sufficient to strongly repress photosynthetic (*RBCS* and *CAB1*) and nitrogen-metabolism (*ASN1*) genes, while causing no or little effect in the wild type. Furthermore, starch-synthesis (*APL3*) and anthocyanin biosynthesis (*CHS*) genes were clearly overinduced in the mutant. Thus, our analyses show that the SR45 protein is involved in the control of sugar-mediated gene activation and repression. While the reduction in photosynthesis-related gene expression is correlated with the HXK1-mediated signaling function (Jang and Sheen, 1994), the effects of glucose on the expression of *ASN1*, *AGPase* and *CHS* are independent of the HXK1 sensor (Xiao et al., 2000), suggesting that SR45 could be involved in both the HXK1-dependent and the HXK1-independent glucose signaling pathways.

The identification of ABA- and ethylene-related mutants in sugar response screens demonstrates the close interaction between sugar and hormonal control of developmental processes. In particular, a mutually antagonistic relationship has been uncovered between glucose and ethylene signaling during early seedling development (Zhou et al., 1998). However, the enhanced sugar sensitivity of the *sr45-1* mutant is not due to ethylene insensitivity, as hypocotyl elongation is equally sensitive to ethylene inhibition in mutant and wild-type seedlings. Moreover, the antagonistic effect of ethylene on the glucose response is still observed in the *sr45-1* mutant. Hence, the mode of action of SR45 in sugar signaling appears to be independent of the ethylene signaling pathway.

Nevertheless, the *sr45-1* mutation leads to altered ABA signaling, providing further support to extensive crosstalk between the glucose and ABA signal transduction pathways. Although not as striking as the glucose response, mutant seedlings show enhanced sensitivity to the hormone during cotyledon development. Previously characterized ABA hypersensitive mutants, such as *era* (*enhanced response to ABA*) *1* (Dekkers et al., 2008), *era3* (Cheng et al., 2002) and *ahg* (*ABA-hypersensitive germination*) *1* (Nishimura et al., 2007), also display glucose oversensitivity. Furthermore, many mutations that lead to altered sugar signaling have previously been found to be allelic to those that affect ABA response or synthesis (Arenas-Huertero et

al., 2000; Laby et al., 2000; Rook et al., 2001), suggesting that sugar responses are directly mediated by ABA via the induction of its biosynthesis and activation of ABA signaling genes. Indeed, we have shown that the *sr45-1* mutant displays enhanced ABA accumulation in response to glucose, which correlates with a marked induction of ABA biosynthesis gene expression. In addition, rescue of glucose inhibition of cotyledon expansion in 3% glucose with the ABA biosynthesis inhibitor fluridone indicates that the glucose-induced rise in ABA levels is required for the growth arrest observed in the mutant. It is therefore clear that SR45-mediated sugar signaling is operating, at least to a certain extent, via regulation of glucose-specific ABA accumulation.

On the other hand, transcript levels of two key ABA signaling genes, *ABI3* and *ABI5*, are markedly over induced by glucose in the *sr45-1* mutant, suggesting that the growth arrest in 3% glucose is mediated by these two transcription factors whose concerted action leads to an ABA-dependent postgermination arrest (Lopez-Molina et al., 2002). The SR45 protein is therefore involved in the transcriptional repression of *ABI3* and *ABI5* in the presence of glucose, probably via downregulation of the levels of ABA, which is known to activate transcription of these genes. Downregulation of *ABI3* and *ABI5* expression may hence underlie the effect of SR45 in counteracting the sugar-mediated growth arrest.

Thus, the role of SR45 in sugar signaling involves downregulation of the ABA pathway, via both reduction of the sensitivity to the phytohormone and a decrease in its endogenous accumulation. Nonetheless, it appears that ABA only partially accounts for the behavior of *sr45-1* in the presence of glucose. In fact, the sugar induced a stronger phenotypical response and higher activation of *ABI3* and *ABI5* transcription than ABA in the *sr45-1* mutant. Moreover, the ABA biosynthesis inhibitor fluridone was unable to fully rescue the mutant's sugar phenotype, suggesting that glucose-specific ABA biosynthesis may not be the sole factor mediating the observed *sr45-1* glucose responses.

The fact that the mutant's over induction of *ABI3* and *ABI5* by glucose is also seen at the pre-mRNA level strongly suggests that the observed variations in gene

expression are due to an increase in transcription rates rather than in mRNA stability. As the evolutionarily conserved SR proteins have been mainly implicated in pre-mRNA splicing and post-splicing activities (such as mRNA stability, export and translation) in various organisms and not in transcriptional control, it is unlikely that *ABI3* and *ABI5* represent direct molecular targets of the SR45 protein. In fact, as pointed out above, it is probable that these two ABA signaling genes are indirectly activated upon glucose-induced ABA accumulation.

In an attempt to gain further insight into the molecular mechanisms of SR45, we compared the splicing patterns of numerous candidate genes in the mutant and Col-0 backgrounds. However, no appreciable changes in alternative splicing were observed either under control conditions or in the presence of glucose for any of the analyzed genes. On one hand, our candidate gene strategy may have been insufficient in the coverage of putative splicing target genes. On the other, it should be noted that the RT-PCR approach employed may fail to detect subtle changes in alternative splicing that could still account for the observed phenotypes. The fact remains, however, that SR protein splicing targets apart from other members of the SR gene family (Lopato et al., 1999; Kalyna et al., 2003; Ali et al., 2007) are still to be identified in plants despite considerable efforts by several groups in recent years. In particular, microarray experiments combined with verification by RT-PCR revealed upregulation of the flowering repressor *FLC* in *sr45-1*, but no distinguishable alteration in the alternative splicing pattern of any flowering gene (Ali et al., 2007). Although SR45 has been shown to possess splicing activity in vitro and affect alternative splicing of five other SR genes (Ali et al., 2007), the possibility that it is also involved in post-splicing activities cannot be excluded. In fact, SR45 is likely to be an ortholog of the human RNPS1 (Zhang and Mount, 2009), a component of the exon junction complex (EJC) (Le Hir et al., 2001), which has major influence in localization, export, surveillance and translation of spliced mRNA (Tange et al., 2004). Further molecular and biochemical characterization of the SR45 splicing factor should reveal whether it plays additional roles in posttranscriptional regulation and help pinpoint the molecular targets underlying its mode of action.

3.5. Material and methods

3.5.1. Plant materials and growth conditions

Seeds from *Arabidopsis thaliana* (L.) Heyhn, ecotype Columbia-0 (Col-0), were surface-sterilized and sown in petri dishes containing Murashige and Skoog (MS) salts (Duchefa Biochemie, Haarlem, The Netherlands), 2.5 mM MES (pH 5.7), 0.5 mM myo-inositol and 0.8% agar. After stratification for 3 days in the dark at 4ºC (to break dormancy), the seeds were transferred to a growth chamber under 16h-photoperiod (80 µmol.m^{-2}.s^{-1} white light) at 22ºC and 60% RH. After 2-3 weeks, plants were transferred to soil in individual pots.

Seeds containing a T-DNA insertion in the *SR45* (At1g16610) gene (insertion line Salk_004132, derived from the Col-0 ecotype) were obtained from the SALK collection (http://signal.salk.edu) and grown as described above. The exact location of the insertion (exon 7, pos. 1967 bp) was verified with *SR45*-specific primers (see Table 3.3) and primers annealing at the left border of the T-DNA. Genotyping by PCR allowed the identification of homozygous lines for the insertion. This mutant line was previously isolated by Ali *et al.* (2007) and named *sr45-1*.

3.5.2. Growth assays

Plants of different genotypes were sown and grown simultaneously under identical conditions. Seeds from fully matured siliques of dehydrated plants of the same age were collected and stored in the dark at room temperature. All assays were performed with seeds from comparable lots stored for 2-18 months.

For germination assays, seeds were surface-sterilized and water-imbibed in the dark for 3 days at 4ºC. After stratification, 80-100 seeds of each genotype were sown in triplicate in petri dishes containing MS medium (MS salts, 2.5 mM MES pH 5.7, 0.5 mM myo-inositol and 0.8% agar), supplemented or not with the appropriate concentrations of D-glucose, mannitol, ABA (mixed isomers, A1049; Sigma, St Louis, MO, USA), ACC (MP-Biomedicals, Eschwege, Germany) or fluridone (Fluka, Steinheim, Germany), before transfer to the growth chamber (16-h photoperiod).

Germination (defined as the protrusion of the radicle through the seed coat), cotyledon greening and cotyledon expansion were scored every day after transfer to the growth chamber, and cotyledon greening and expansion rates were calculated over the total of germinated seeds. Average percentages were calculated with SEs of the triplicates.

For assessment of hypocotyl elongation, 20-50 seeds of each genotype were surface-sterilized and stratified as described above, plated in MS plates supplemented or not with the appropriate concentrations of glucose, mannitol or ACC and grown vertically in complete darkness at 22ºC for 6 days. Etiolated seedlings were illuminated for 12h before hypocotyl length measurements using ImageJ sofware (http://rsbweb.nih.gov/ij).

All assays were repeated at least three times with similar results.

3.5.3. Isolation of *SR45* cDNA and complementation analysis

First-strand *SR45* cDNA was obtained from total RNA extracted from 1-3 week old wild-type seedlings using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and a poly-T primer. Primers 5'-TGGTTGGCGCGCCATGGCGAAACCAAGTCGTGG-3' and 5'-ACCCATTAATTAATTAAGTTTTACGAGGTGGAGGTGG-3' — which introduced an *AscI* and *PacI* restriction site, respectively — were used for PCR amplification and the DNA fragments corresponding to the coding sequences of the two *SR45* isoforms were cloned into the binary pBA002 vector under the control of the 35S promoter and sequenced. Each of these constructs was then introduced into *Agrobacterium tumefaciens* for floral dip transformation (Clough and Bent, 1998) of homozygous *sr45-1* plants. T1 transformants were selected on BASTA-containing medium, grown to maturity, and selfed. At least two independent transgenic lines were selected from each transformation. All phenotypic analysis was carried out in T3 plants.

3.5.4. RNA extraction and RT-PCR analysis

Col-0 and *sr45-1* seedlings were grown in MS plates supplemented or not with 3% glucose, 3% mannitol or 1 µM ABA. Plant material was harvested at day 7 after stratification for glucose-responsive gene expression analysis, at the same developmental stage (~50% cotyledon expansion) for analysis of ABA biosynthesis genes, and 4 days post stratification for analysis of *ABI3* and *ABI5* expression. For *SR45* expression analysis in transgenic complementation lines, 2-week old plants grown in MS medium were used. Total RNA was extracted from whole seedlings using the innuPREP Plant RNA kit (Analytik Jena BioSolutions, Jena, Germany) following the protocol provided. All RNA samples were digested with DNaseI (Promega, Madison, WI, USA) and phenol-chloroform purified before reverse transcription with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. *SR45*, glucose-responsive genes, ABA biosynthesis and signaling genes, and the housekeeping *cyclophilin (CYC), ubiquitin10 (UBQ10)* and *actin* (*ACT*) genes were amplified by PCR with the gene-specific primers shown in Table 3.3. Preliminary PCRs were carried out with different cycles to determine the linear range of amplification. Based on these analyses, 20-35 cycles were chosen for DNA detection, depending on the gene (Table 3.3). Amplified DNA fragments were separated in 1% agarose gels. Band intensities were quantified using the NIH IMAGEJ program (http://rsb.info.nih.gov/ij/), normalizing transcript levels to *CYC*, *UBQ10* or *ACT* and setting the expression of each gene in the wild type under control conditions to one.

To detect pre-mRNA, reverse transcription was carried out using Random Primers (Invitrogen, Carlsbad, CA, USA) instead of a poly-T primer, and PCR amplification was performed with one primer annealing at an intronic sequence.

Table 3.3. Sequences of the gene-specific primers used in RT-PCR analyses.

Gene name	Locus ID	Primers	# cycles
SR45	At1g16610	Fwd: 5'-AGCTCAGCTCTACATGGATGG-3' Rev: 5'-AGGAGACCTTCTTCGAACAGG-3'	25
RBCS	At5g38410	Fwd: 5'-GGCTAAGGAAGTTGACTACC-3' Rev: 5'-ACTTCCTTCAACACTTGAGC-3'	20
CAB1	At1g29930	Fwd: 5'-ATCTGAAGTCCTTGGAAGCGG-3' Rev: 5'-CCAAACATAGAGAACATAGCC-3'	25
ASN1	At3g47340	Fwd: 5'-AGGTGCGGACGAGATCTTTGG-3' Rev: 5'-GTTGTCAATTGCCTTAAGTGG-3'	28
APL3	At4g39210	Fwd: 5'-CTGTGGGTGTTTGAGGATGC-3' Rev: 5'-GATACCGCCATGTCAGAAGC-3'	25
CHS	At5G13930	Fwd: 5'-TCAAGCGTCTCATGATGTACC-3' Rev: 5'-GTCGCCCTCATCTTCTCTTCC-3'	25
MYB75	At1g56650	Fwd: 5'-GCTGGGCTAAACCGGTGCAGG-3' Rev: 5'-TTCTGTTGTCGTCGCTTCAGG-3'	30
MYB90	At1g66390	Fwd: 5'-TCAAGTTCCTTTGAGAGCTGG-3' Rev: 5'-TCCCCCAGTAAATTCTCCAAC-3'	30
ABI3	At3g24650	Fwd: 5'-CTTCAACGATGAAAAGCTTGC-3' Rev: 5'-GCTGAGGTGTCAAAGAACTCG-3'	28
*ABI3**		Fwd: 5'-CAGCAGAACCAAACCCAAATC-3' Rev: 5'-TTAGAAAATGGAAAGCAAAGG-3'	35
ABI5	At2g36270	Fwd: 5'-TGGTTCTAGAGGCAACGAAGA-3' Rev: 5'-CCTCCATAGCAAACACCTGCC-3'	28
*ABI5**		Rev: 5'-AAAAGGAATGAAGAAGGTCTC-3'	35
UBQT10	At4g05320	Fwd: 5'-GATCTTTGCCGGAAAACAATTGG-3' Rev: 5'-TAGAAAGAAAGAGATAACAGG-3'	23
Cyclophilin	At4g38740	Fwd: 5'-GTCTGATAGAGATCTCACGT-3' Rev: 5'-AATCGGCAACAACAACAGGC-3'	25
ACT	At3g18780	Fwd: 5'-TTTGCAGGAGATGATGCTCCC-3' Rev: 5'-GTCTTTGAGGTTTCCATCTCC-3'	25

* primers used for pre-mRNA detection

3.5.5. Abscisic acid content determination

For quantification of ABA content, whole seedlings grown in MS media with or without 3% glucose were collected at the same developmental stage (~50% cotyledon expansion). The plant material (30-60 mg) was grounded in liquid nitrogen and then homogenized in 1 mL of ABA-extraction buffer (10 mg.L^{-1} butylated hydroxytoluene, 20 mL.L^{-1} acetic acid, 90% methanol). Extraction was carried out overnight with constant shaking at 4ºC and the supernatant subsequently collected and evaporated to dryness

using a speed-vac. ABA levels were then quantified with the Phytodetek-ABA-kit (AGDIA Inc., Elkhart, IN) using the protocol provided. Mixed ABA isomers (A1049; Sigma, St. Louis, MO, USA) were used as a standard.

R. Carvalho was the main responsible for the experimental work and data analysis.

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CHAPTER 4

The Arabidopsis SR45 splicing factor, a negative regulator of sugar signaling, targets alternative splicing of the *5PTase13* gene and modulates SnRK1.1 levels

4.1. Abstract

We have previously reported that the plant-specific SR45 splicing factor defines a negative regulator of glucose signaling in Arabidopsis. Here we show that disruption of the gene encoding hexokinase1 (HXK1) does not suppress the glucose phenotypes of the *sr45-1* loss-of-function mutant, indicating that the mode of action of SR45 in sugar signaling is independent of this conserved glucose sensor. However, western blot analyses revealed that in the presence of sugar *sr45-1* contains elevated levels of SnRK1.1 and SnRK1.2, two closely-related protein kinases involved in the coordination of sugar and stress responses. The enhanced SnRK1.1 levels in *sr45-1* were suppressed by the presence of a proteasome inhibitor, suggesting a role for SR45 in destabilizing the SnRK1.1 protein in response to sugars. Importantly, the *sr45-1* mutation caused changes in alternative splicing of the *5PTase13* gene, which encodes a myoinositol polyphosphate 5-phosphatase previously shown to interact with and regulate the stability of SnRK1.1 in vitro, thus providing a mechanistic link between SR45 function and the modulation of SnRK1.1 levels in response to sugars. Finally, we made use of a high-resolution RT-PCR alternative splicing panel to identify additional physiological targets of the SR45 splicing factor. Out of 242 genes analyzed, 93 showed significant changes in their alternatively-spliced transcripts as a result of the *sr45-1* mutation, including *SnRK1.2*, a dormancy/auxin-associated gene, and *SR45* itself.

4.2. Introduction

SR45 is an RNA-binding protein highly conserved in the plant kingdom, which appears to be exclusive to flowering plants (Ali et al., 2007). It has been shown to function as an essential splicing factor, as it is able to complement a mammalian extract deficient in SR proteins (Ali et al., 2007), and hence had been regarded as a member of this important family of splicing regulators. However, the recently revised nomenclature of mammalian (Manley and Krainer, 2010) and plant (Barta et al., 2010)

SR proteins is based entirely on their sequence properties, and owing to its highly atypical SR protein structure — a single RNA recognition motif (RRM) flanked by two arginine/serine-rich (RS) domains, instead of one or two N-terminal RRMs and a downstream RS domain — *SR45* has now been excluded from this gene family. It is a close homolog of the human RNPS1 protein (Pendle et al., 2005; Zhang and Mount, 2009), a general and versatile splicing activator (Mayeda et al., 1999; Sakashita et al., 2004) and component of the exon junction complex (Le Hir et al., 2001). Interestingly, RNPS1 has been found to be a nucleocytoplasmic shuttling protein implicated in other aspects of RNA metabolism, such as nuclear export and nonsense mediated decay (Lykke-Andersen et al., 2001), as well as in alleviating genome instability (Li et al., 2007).

The *Arabidopsis thaliana* SR45 was first identified in a yeast two-hybrid screen as a binding partner of U1-70K (Golovkin and Reddy, 1999), a U1 small nuclear ribonucleoprotein-specific protein with key functions in early spliceosome assembly. The in vivo interaction between these two proteins was later confirmed using co-localization studies and bimolecular fluorescence complementation assays and shown to require both RS domains of SR45 (Ali et al., 2008). Moreover, SR45 interacts with at least another Arabidopsis SR protein, SR33, and AFC2, a LAMMER-type protein kinase, which is able to phosphorylate SR45 in vitro (Golovkin and Reddy, 1999). All subcellular localization studies reported to date have found SR45 to be confined to the nucleus, either diffusely distributed in the nucleoplasm or concentrated in speckles, with its subnuclear dynamics being regulated by ATP, phosphorylation and transcription (Ali et al., 2003; Ali and Reddy, 2006). Like many other splicing-related components, the Arabidopsis *SR45* gene itself undergoes alternative splicing, generating two splice variants that differ by an in-frame 21-nucleotide sequence as a result of an alternative 3' splice site selection event (Palusa et al., 2007; Zhang and Mount, 2009).

Previous functional analyses of *SR45* stem solely from the characterization of the Arabidopsis T-DNA insertion mutant, *sr45-1*. Reddy and co-workers first reported that

sr45-1 displays pleiotropic developmental defects, including reduced plant size, delayed flowering, altered leaf and flower morphology, as well as slower root growth (Ali et al., 2007). Remarkably, expression of the longest *SR45* splice variant in the knockout background only rescues the floral morphology phenotype, while the shortest complements exclusively the delay in root growth, thus demonstrating that the two alternatively-spliced isoforms of *SR45* fulfill distinct physiological roles (Zhang and Mount, 2009). More recently, a third *SR45* splice variant including 33-nucleotides that are absent from the other two splice variants has been annotated, but its expression pattern and functional significance remain unknown. In addition to its roles in normal plant development, we have recently shown that the SR45 protein negatively regulates glucose signaling during early seedling growth in Arabidopsis by downregulating abscisic acid (ABA) biosynthesis and signaling (Carvalho et al., 2010).

Higher plants are able to transduce sugar signals in order to adjust developmental programs, maintain energy homeostasis and achieve stress tolerance in response to metabolic and environmental fluctuations. Glucose, in addition to being a carbon and energy source, plays an important regulatory role as a central signaling molecule, modulating gene expression and influencing a variety of processes such as germination, early seedling development, flowering and senescence (Koch, 1996; Gibson, 2005; Rolland et al., 2006). The glycolytic enzyme hexokinase (HXK) is an evolutionarily conserved glucose sensor, operating as a dual-function enzyme with both catalytic and regulatory roles also in plants. Indeed, the characterization of *HXK* antisense and overexpression lines in Arabidopsis and tomato (Jang et al., 1997; Dai et al., 1999), and particularly of an Arabidopsis null mutant together with catalytically inactive *HXK1* alleles (Moore et al., 2003), have established HXK1 as a true glucose sensor in plants, while demonstrating that glucose signaling can be uncoupled from glucose metabolism. In addition to HXK-mediated signaling, studies of glucose-responsive gene expression in sense and antisense *HXK1* Arabidopsis transgenic lines have suggested the existence of a HXK1-independent pathway for glucose signal transduction in plants (Xiao et al., 2000). More recently, an *Arabidopsis thaliana* serine/threonine kinase, SnRK1, was described as a sugar sensor and central

integrator of a transcription network for stress and energy signaling (Baena-Gonzalez et al., 2007), with a subsequent study reporting its involvement in sugar and ABA signaling (Jossier et al., 2009).

The molecular mechanisms underlying the biological roles of the SR45 protein remain poorly understood. Apart from five Arabidopsis SR proteins, no SR45 splicing targets have been identified to date, leading to the suggestion that the regulation of plant developmental processes occurs indirectly via the modulation of SR protein isoforms, which would in turn affect splicing of downstream genes (Ali et al., 2007). Here, we show that the mode of action of SR45 in sugar signaling is HXK1-independent, but involves glucose-responsive modulation of the degradation of the SnRK1.1 protein kinase. Importantly, SR45 affects the splicing pattern of the *5PTase13* gene, which encodes an inositol polyphosphate 5-phosphatase previously shown to interact with and regulate the stability of SnRK1.1 in vitro (Ananieva et al., 2008), providing a mechanistic link between SR45 function and the modulation of SnRK1.1 levels in response to sugars. Finally, using a high-resolution RT-PCR alternative splicing panel, we pinpoint additional splicing targets of this RNA-binding protein.

4.3 Results

4.3.1. SR45 control of glucose responses is independent of HXK1-mediated sugar signaling

Our previous finding that the *sr45-1* mutant is hypersensitive to glucose during early seedling development (Carvalho et al., 2010) prompted us to investigate the dependency of this phenotype on the HXK1-mediated signal transduction pathway. To this end, we first examined whether the *sr45-1* mutation affects expression of the *HXK1* gene (At4g29130) in both young seedlings and adult leaves of *Arabidopsis thaliana*. As shown in Figure 4.1A, in agreement with a previous global microarray-based transcriptome analysis (Price et al., 2004), exogenous glucose markedly induced *HXK1* expression in young Arabidopsis seedlings. Moreover, we found that

HXK1 transcript levels were also upregulated by glucose treatment of detached leaves (Figure 4.1A). However, glucose regulation of the *HXK1* gene in both these tissues was unaffected by the *sr45-1* mutation (Figure 4.1A).
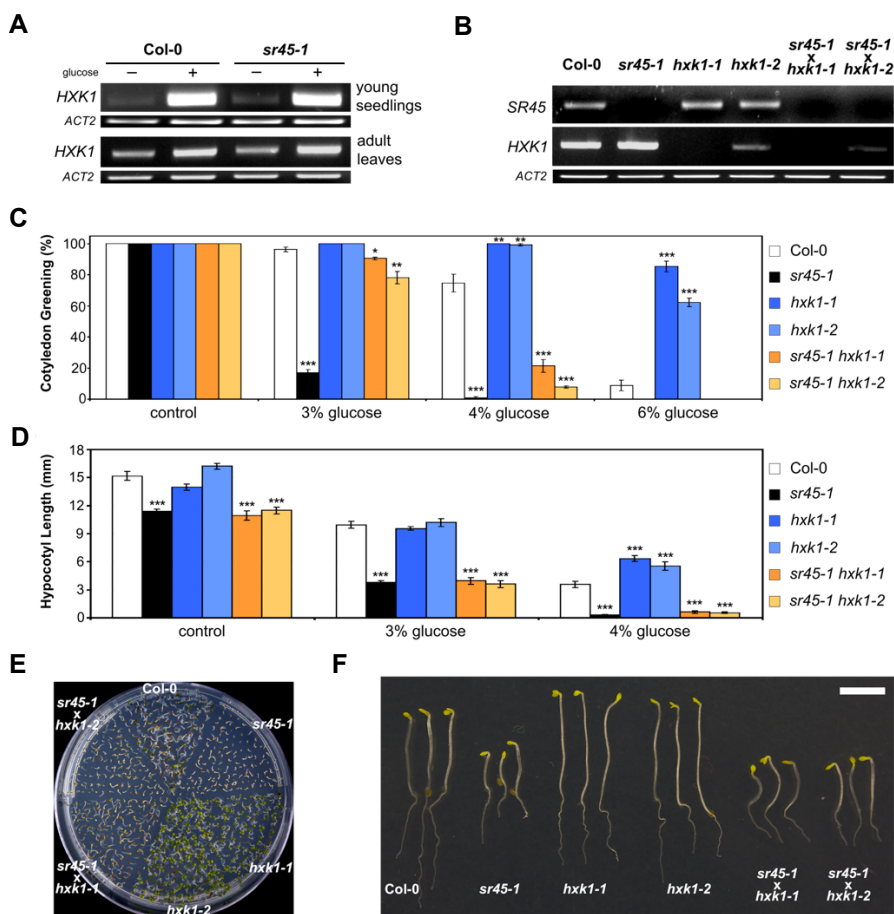

Figure 4.1. HXK1 dependency of the *sr45-1* glucose phenotypes.
A. RT-PCR analysis of *HXK1* transcript levels in wild-type (Col-0) and mutant (*sr45-1*) plants, both in young seedlings grown in the absence or presence of 3% glucose and detached leaves treated or not with 1.5% glucose. The *actin2* gene was used as a loading control. **B.** RT-PCR analysis of *SR45* and *HXK1* transcript levels in Col-0, *sr45-1*, two insertion mutant alleles for *HXK1 (hxk1-1* and *hxk1-2)* and the corresponding *sr45-1 hxk1* double mutant plants. *Actin2* is shown as a loading control. **C.** Cotyledon greening rates, scored 7 d after stratification, of Col-0, *sr45-1*, two *hxk1* mutant alleles, and *sr45-1 hxk1*

double mutant seedlings grown under control conditions or in the presence of different glucose concentrations (means ± SE, *n*=3). Asterisks indicate significant differences (*, P < 0.05; **, P < 0.01; ***, P < 0.001) from the corresponding wild type according to a Student's *t*-test. **D.** Hypocotyl length, measured 7 d after stratification, of Col-0, *sr45-1*, *hxk1-1*, *hxk1-2*, *sr45-1 hxk1-1* and *sr45-1 hxk1-2* seedlings grown in the dark under control conditions or in the presence of 3% or 4% glucose (means ± SE, *n*=30-60). Asterisks indicate significant differences (***, P < 0.001) from the corresponding wild type according to a Student's *t*-test. **E.** and **F.** Representative images of Col-0, *sr45-1*, *hxk1-1*, *hxk1-2*, *sr45-1 hxk1-1* and *sr45-1 hxk1-2* seedlings grown in the presence of 5% glucose for 5 d **(E)** or in the dark in the presence of 3.5% glucose for 7 d **(F)** Bar=6 mm.

To conclusively assess whether glucose hypersensitivity of the *sr45-1* mutant is linked to HXK1-mediated glucose signaling, we used a genetic approach and generated double mutants in which both the *SR45* and *HXK1* genes are disrupted. The *sr45-1* mutant is in the Col-0 background, whereas the most extensively characterized null mutant for *HXK1*, *gin2-1*, was isolated via EMS mutagenesis of L*er* ecotype seeds (Moore et al., 2003). Thus, to obviate problems in the double mutant phenotypical analysis arising from different parental ecotypes, instead of the original *gin2-1* mutant we made use of two Col-0 background mutant lines for *HXK1*. Both *hxk1-1* (SALK_018086) and *hxk1-2* (SALK_070739) harbor T-DNA insertions in the first intron of the *HXK1* gene. However, RT-PCR analysis using a primer pair flanking the insertions revealed that the *HXK1* transcript is below detectable levels in *hxk1-1*, suggesting that this is a true null mutant, while *hxk1-2* exhibits a reduction in the levels of *HXK1* mRNA (Figure 4.1B). Nevertheless, consistent with an earlier report (Aki et al., 2007), our results indicate that both these single mutant lines display glucose insensitivity during early seedling development (Figures 4.1C-F), as expected from the loss of function of a critical component in the sensing of glucose levels.

Importantly, disruption of the *HXK1* gene did not suppress the glucose oversensitive phenotypes conferred by the *sr45-1* mutation. In the presence of sugar, *sr45-1* seedlings exhibit an early growth arrest, with cotyledon development being significantly impaired in comparison to the Col-0 wild type (Carvalho et al., 2010) (Figures 4.1C and 4.1E). As seen in Figures 4.1C and 4.1E, both the *sr45-1 hxk1-1*

and the *sr45-1 hxk1-2* double mutants also displayed reduced cotyledon greening and expansion when grown in the presence of glucose concentrations ranging from 3% to 6%. Furthermore, the *sr45-1* mutant, which is affected in general development and hence shows shorter hypocotyls in the absence of sugar, displays increased sensitivity to the glucose inhibitory effects on hypocotyl elongation of dark-grown seedlings (Carvalho et al., 2010) (Figure. 4.1D and 4.1F). Similarly, the glucose-dependent reduction in hypocotyl elongation observed in the wild type was enhanced in *sr45-1 hxk1-1* and *sr45-1 hxk1-2* etiolated seedlings (Figures 4.1D and 4.1F). Thus, *sr45-1 hxk1* double mutants retain hypersensitivity to glucose both at the level of cotyledon development and hypocotyl elongation in the dark. Together, these results clearly demonstrate that the role of the SR45 splicing factor in sugar signaling is HXK1-independent.

4.3.2. SR45 modulates SnRK1.1 stability in response to glucose

The conclusion that the *SR45* and *HXK1* genes are operating in distinct pathways elicited the search for an alternative sensing/signaling component governing the mode of action of the SR45 splicing factor in sugar responses. In plants, the evolutionarily conserved SNF1-related kinases (SnRK1s) are known to control carbohydrate metabolism, and the *Arabidopsis thaliana* SnRK1.1 and SnRK1.2 (also known as KIN10/At3g01090 and KIN11/At3g29160, respectively) have been identified as central integrators of a transcription network for stress and energy signaling (Baena-Gonzalez et al., 2007). Importantly, a subsequent study reported that overexpression of SnRK1.1 results in hypersensitivity to both glucose and ABA (Jossier et al., 2009), a strikingly similar phenotype to that caused by loss of *SR45* function. This prompted us to analyze the expression and splicing pattern of *SnRK1.1* in the *sr45-1* mutant.

The *SnRK1.1* gene encodes three mRNAs arising from selection of alternative 5' splice sites in the first intron (Figure 4.2A). RT-PCR analysis using primers designed to detect all three transcripts allowed significant amplification of only the first splice variant, SV1, which appeared highly and similarly expressed in wild-type and mutant plants both under control and glucose conditions (Figure 4.2A). However, primers

designed to amplify exclusively the other two alternative transcripts enabled detection of SV2 and SV3, which were also expressed at similar levels in all samples analyzed (Figure 4.2A). Thus, at least under the conditions tested, SV1 is largely the most expressed *SnRK1.1* transcript, with our results revealing no SR45-induced changes in the expression or splicing pattern of the *SnRK1.1* gene.
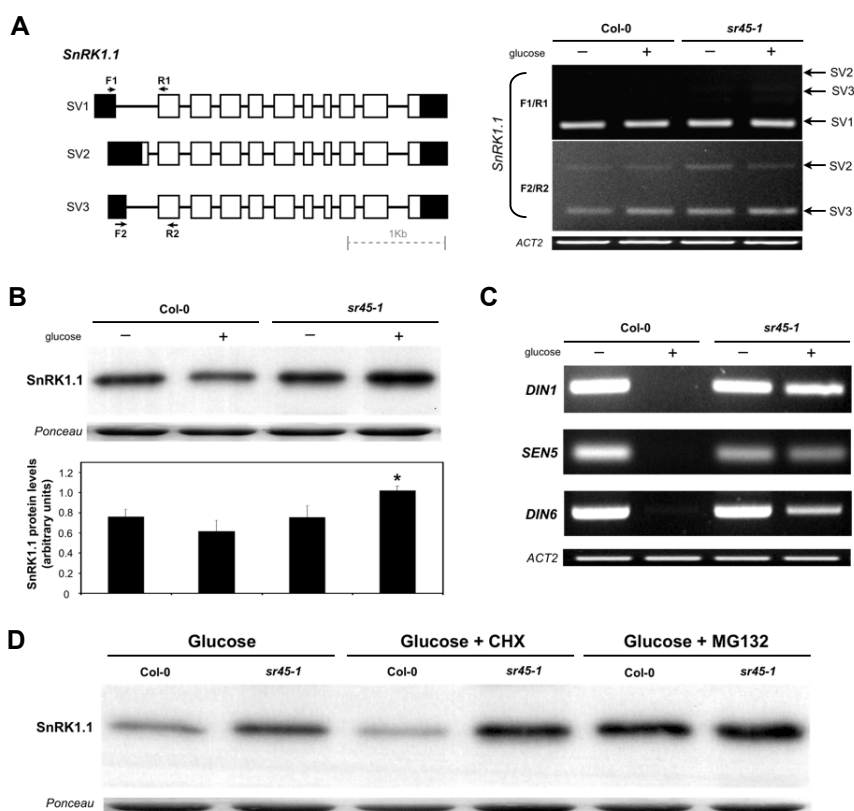

Figure 4.2. SR45 modulation of SnRK1.1 protein levels.

A. Schematic diagram of the three splice variants (SVs) produced by the *SnRK1.1* gene (boxes represent exons with UTRs in black, lines represent introns, while arrows indicate the location of the F1, R1, F2, R2 primers), and RT-PCR analysis of *SnRK1.1* transcript levels in wild-type (Col-0) and mutant (*sr45-1*) leaves incubated for 6h in the absence or presence of 1.5% glucose. The *actin2* gene was used as a loading control. **B.** Western blot analysis of SnRK1.1 levels in Col-0 and *sr45-1* leaves incubated in the absence or presence of 1.5% glucose (Ponceau-stained membrane is shown as a loading control). Bands

were quantified and relative protein levels determined using the Ponceau control as a reference. The blot image is representative of four independent experiments and the bar graph shows means ± SE of SnRK1.1 levels in all assays (*n*=4). The asterisk indicates a significantly different value from the wild type under both control and glucose conditions (P < 0.05, Student's *t*-test). **C.** RT-PCR analysis of the transcript levels of SnRK1.1-activated marker genes *DIN1 (*At4g35770), *SEN5 (*At3g15450) and *DIN6 (*At3g47340) in Col-0 and *sr45-1* leaves treated or not with 1.5% glucose. The *actin2* gene was used as a loading control. **D.** Western blot analysis of SnRK1.1 protein levels in leaves treated with 1.5% glucose in the absence or presence of 100 μM of the protein synthesis inhibitor cycloheximide (CHX) or of 50 μM of the proteosome inhibitor MG132.

We next turned to examine potential alterations in SnRK1.1 protein levels. Importantly, despite unchanged transcript levels but consistent with the fact that overexpression of *SnRK1.1* phenocopies the *sr45-1* mutant (Jossier et al., 2009), western blotting analysis indicated that *sr45-1* contains significantly higher amounts of the SnRK1.1 protein kinase than the wild type, particularly in the presence of glucose (Figure 4.2B). Very similar results were obtained when SnRK1.2 levels were assessed (Figure 4.3A). Moreover, the difference in the amounts of SnRK1.1 between wild-type and mutant plants was not observed when both genotypes were treated with equimolar concentrations of sorbitol (Figure 4.3B), showing that the effect of the *sr45-1* mutation on SnRK1.1 levels is glucose-specific and not solely due to the osmotic stress imposed by the sugar. In agreement with enhanced SnRK1.1 protein levels in the glucose-treated *sr45-1* mutant, genes whose transcription is activated by SnRK1.1, such as *DIN1* (also known as *SEN1*/At4g35770), *SEN5* (At3g15450) and *DIN6* (also known as *ASN1*/At3g47340), appear markedly overexpressed in *sr45-1* under glucose conditions. Although these dark-induced genes are expressed only at basal levels in the light, an increased number of PCR cycles revealed that the three SnRK1.1 targets were as expected dramatically repressed by glucose in the wild type, but only slightly downregulated by the sugar in mutant plants (Figure 4.2C).
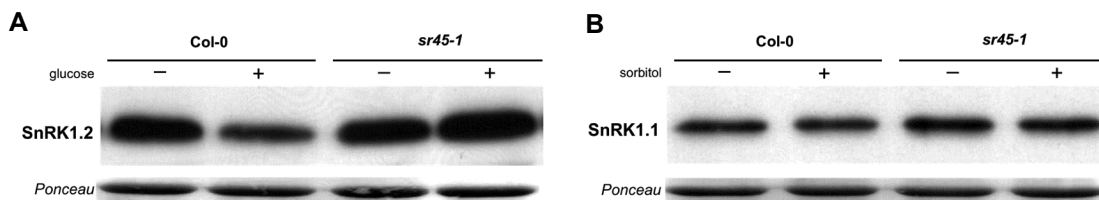
Figure 4.3. Effect of glucose and sorbitol on SnRK1.2 and SnRK1.1 levels, respectively, in wild-type and *sr45-1* mutant plants.

A. Western blot analysis of SnRK1.2 protein levels in wild-type (Col-0) and mutant (*sr45-1*) leaves incubated for 6h in the absence or presence of 1.5% glucose (Ponceau-stained membrane is shown as a loading control). **B.** Western blot analysis of SnRK1.1 protein levels in Col-0 and *sr45-1* leaves incubated for 6h in the absence or presence of 1.5% sorbitol (Ponceau-stained membrane is shown as a loading control).

Finally, we investigated whether the elevated levels of SnRK1.1 detected in the *sr45-1* mutant are due to enhanced protein biosynthesis or reduced proteolysis. As shown in Figure 4.2D, the difference in SnRK1.1 amounts between mutant and wild type observed under glucose conditions was unaltered in the presence of the protein synthesis inhibitor cycloheximide (CHX). By contrast, treatment with MG132, a potent proteasomal inhibitor, resulted in increased SnRK1.1 content in wild-type plants and suppressed the enhanced *sr45-1* SnRK1.1 levels (Figure 4.2D), strongly suggesting a role for SR45 in destabilizing the SnRK1.1 protein in response to sugars. Thus, the SR45 splicing factor appears to act through glucose-responsive modulation of the degradation of a conserved protein kinase known to sense stress-associated energy-deprivation.

4.3.3. SR45 targets alternative splicing of the *5PTase13* gene

Two components have been previously implicated in the regulation of the stability of the SnRK1.1 protein kinase. Pleiotropic regulatory locus 1 (PRL1), a nuclear WD40 repeat protein that binds both SnRK1.1 and SnRK1.2 inhibiting their protein kinase activity in vitro (Bhalerao et al., 1999), has been suggested to act as the substrate receptor for the degradation of SnRK1.1 by a cullin 4 E3 ligase in Arabidopsis (Lee et

al., 2008). On the other hand, a myoinositol polyphosphate 5-phoshatase, 5PTase13, was also found to interact via its WD40 domain with SnRK1.1 and to modulate the amounts of this protein that are targeted to proteasomal degradation (Ananieva et al., 2008). Thus, the transcripts encoding these two Arabidopsis WD proteins represented prime candidates for functional targets of the SR45 splicing factor.

The current genome annotation indicates that the *PRL1* gene (At4g15900) produces a single mRNA, whose expression was found to be unchanged in the *sr45-1* mutant (data not shown). However, by cloning and sequencing its cDNAs, we confirmed that the Arabidopsis *5PTase13* gene (At1g05630) generates two distinct splice variants. While one derives from constitutive splicing of the pre-mRNA, retention of the gene's 102-bp intron 6 gives rise to a longer alternative transcript (Figure 4.4A). As this alternatively-spliced fragment is in frame, the two predicted 5PTase13 isoforms differ only in 34 amino acids, with no recognizable protein domain being included in this region.

Importantly, although no significant differences in total gene expression were detected, we have found that the splicing pattern of the *5PTase13* gene is markedly altered in the *sr45-1* mutant. As seen in Figure 4.4B, RT-PCR analysis using primers designed to detect simultaneously both *5PTase13* splice variants revealed that the abundance of the longer splice variant (SV1) is considerably higher in the mutant. In fact, quantification of the amplified bands and calculation of the ratio between the two alternative mRNAs indicated that while the shorter transcript (SV2) is nearly four times more expressed than SV1 in the wild type, it is only about 1.5 fold more abundant in *sr45-1* plants (Figure 4.4B). Therefore, loss of SR45 function results in enhanced retention of a short intron in the *5PTase*13 gene. These results clearly show that the SR45 protein regulates alternative splicing of the pre-mRNA encoding the 5PTase13 inositol 5-phosphatase, uncovering a potential mechanistic link between SR45 function and the modulation of SnRK1.1 levels in response to sugars.

A

B
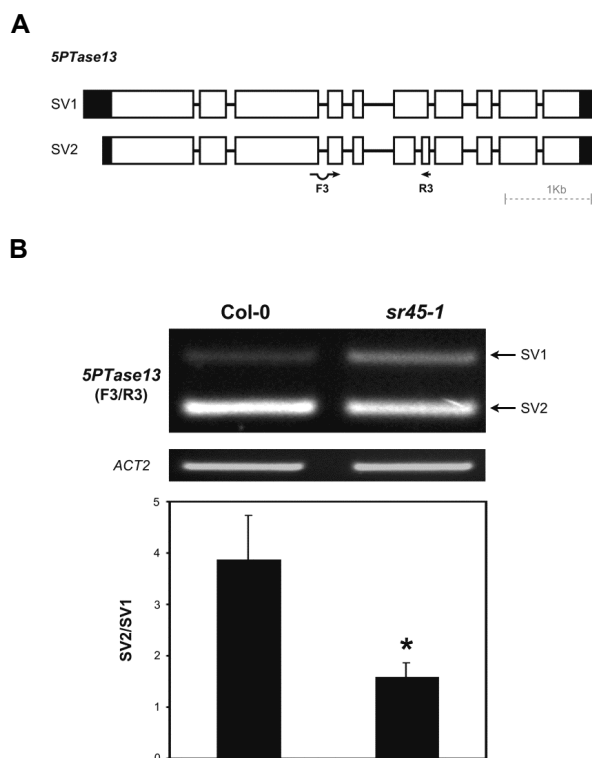
Figure 4.4. SR45 regulation of alternative splicing of the *5PTase13* pre-mRNA.

A. Schematic diagram of the two splice variants (SVs) produced by the *5PTase13* gene. Boxes represent exons with UTRs in black, lines represent introns, and arrows indicate the location of the F3 and R3 primers used in (B). **B.** RT-PCR analysis of *5PTase13* transcript levels in Col-0 and *sr45-1* leaves incubated in the presence of 1.5% glucose (*Actin2* is shown as a loading control). Bands were quantified and relative transcript levels determined using the *Actin2* control as a reference. The histogram shows the SV2/SV1 ratio and is representative of four independent experiments (means ± SE, *n*=4). The asterisk indicates a significantly different value from the wild type (P < 0.01, Student's *t*-test).

4.3.4. SR45 controls alternative splicing of its own pre-mRNA

In order to identify additional splicing targets of the SR45 protein, we compared multiple alternative splicing events in wild-type and *sr45-1* mutant seedlings grown in the absence or presence of 3% glucose using a custom high-resolution RT-PCR panel. This alternative splicing panel was initially developed by Simpson et al. (2008) and expanded to currently include 524 alternative splicing events in 262 Arabidopsis genes (see Table A in Appendix I). For 20 of the genes in the panel only one transcript was

detected, indicating that in these cases the alternative splicing events supported by annotated sequences are either extremely rare or do not occur in the tissue and developmental stage analyzed. As they do not provide alternative splicing information in the plant material tested, these genes were not included in further analyses. The remaining 242 genes encode transcription factors (91), proteins involved in RNA binding and processing (64), stress-related factors (41), proteins regulating flowering time (11) and stomatal ABA signaling (5), as well as other miscellaneous proteins (30). Of the 498 alternative splicing events analyzed in these genes, 179 and 80 involve the selection of alternative 3' and 5' splice sites, respectively, 47 and 22 represent intron retention and exon skipping events, respectively, and two involve both alternative 5' and 3' splice site selection, while 168 events are still to be identified.

Among the 242 genes analyzed, 93 displayed significant ($P < 0.05$) changes in the ratio of alternatively-spliced transcripts of over 3% between wild-type and *sr45-1* plants grown under control conditions (Table B in Appendix I). Using the same criteria, the presence of glucose in the growth media altered the splicing pattern of 82 genes in the wild type (Table C in Appendix I) and 81 genes in the *sr45-1* mutant (Table D in Appendix I). We first asked whether either the *sr45-1* mutation or glucose treatment would preferentially affect the splicing pattern of genes involved in specific functions or a particular type of alternative splicing event (Figure 4.5). While the functional distribution of the genes affected by the *sr45-1* mutation or by glucose treatment in either wild-type or mutant plants was strikingly similar to that of the genes included in the RT-PCR panel (Figure 4.5A), both the mutation and glucose appeared to preferentially affect the selection 3' and 5' splice sites as well as exon skipping events at the expense of unidentified alternative splicing events (Figure 4.5B). However, as with the gene functional analysis, the differences between the alternative splicing event type distribution profiles were not statistically significant ($P > 0.05$, Chi-squared test with Bonferroni correction for multiple testing). Thus, our data do not support any specific gene function or event type bias in the effects of the *sr45-1* mutation and glucose on alternative splicing.

A

B
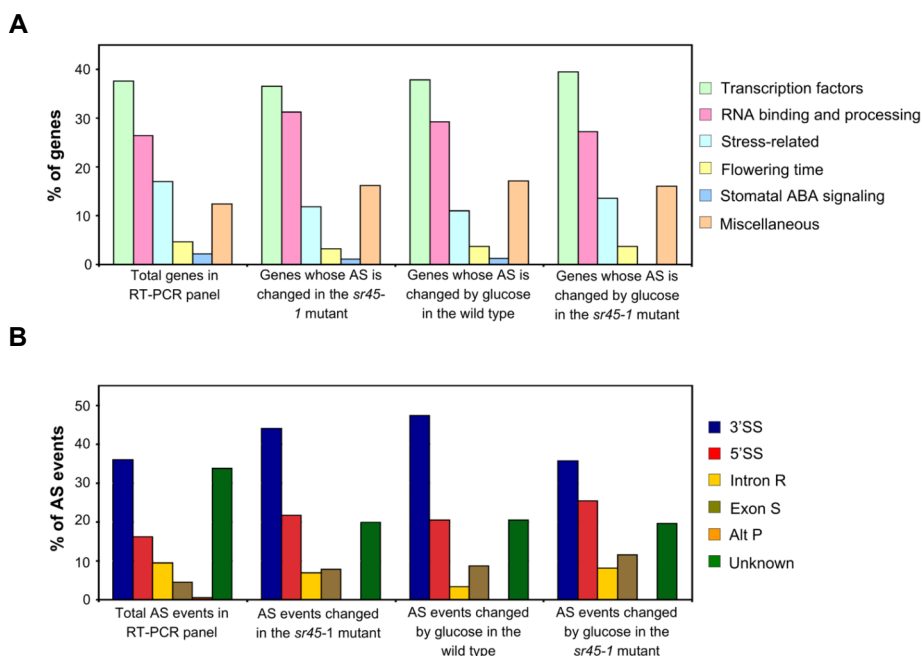
Figure 4.5. Distribution profiles for gene functional category and alternative splicing (AS) event type of the targets identified by the AS RT-PCR panel.
A. Percentage functional distribution of the total genes analyzed by the RT-PCR panel and of the genes showing splicing pattern changes between the wild type and the *sr45-1* mutant, the wild type grown under control and glucose conditions, or the *sr45-1* mutant grown under control and glucose conditions. **B.** Percentage distribution of the different types of AS events in the total AS events analyzed by the RT-PCR panel and in the AS events showing changes between the wild type and the *sr45-1* mutant, the wild type grown under control and glucose conditions, or the *sr45-1* mutant grown under control and glucose conditions. 5'SS, alternative 5' splice site; 3'SS, alternative 3' splice site; Exon S, exon skipping; Intron R, intron retention; Alt P, events involving both alternative 5' and 3' splice sites.
Statistical analysis indicated no significant changes between profiles ($P > 0.05$, Chi-squared test with Bonferroni correction for multiple testing).

Interestingly, we found that the splicing pattern of the *SR45* gene was altered in the *sr45-1* mutant. This loss-of-function mutant, which harbors a T-DNA insertion in the seventh exon of the *SR45* coding sequence, has been reported to express a truncated transcript upstream of the insertion, albeit at considerably lower levels than the wild-type full-length mRNA (Ali et al., 2007). Moreover, selection of alternative 3' splice sites in the gene's sixth intron generates two *SR45* transcripts that differ in 21

nucleotides (Palusa et al., 2007; Zhang and Mount, 2009). As the SR45-specific primer pair used in the RT-PCR panel is located upstream of the T-DNA insertion site and flanks the alternative 3' splice sites, we were able to analyze the effects of the *sr45-1* mutation and glucose on the proportion of the two *SR45* splice variants. The *sr45-1* mutant showed a relative abundance of the shorter *SR45* splice variant about 9% higher when compared to the wild type (Figure 4.6A, Table B in Appendix I), indicating that loss of SR45 function enhances distal 3' splice site usage in intron 6 of the *SR45* pre-mRNA. While the presence of glucose had a marginal effect in wild-type seedlings (Table C in Appendix I), it further increased the proportion of the shorter transcript by approximately 8% in the mutant (Figure 4.6A, Table D in Appendix I), showing that the sugar promotes usage of the distal 3' splice site, particularly in the absence of SR45 function. Thus, our results indicate that glucose and SR45 have opposite effects on processing of the *SR45* pre-mRNA, with the splicing factor favoring expression of the transcript encoding its full-length protein.
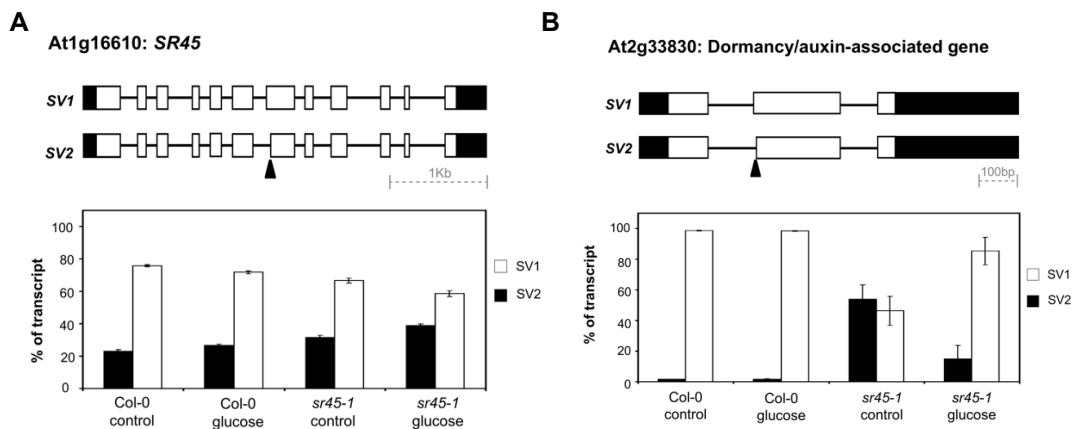

Figure 4.6. The effect of the *sr45-1* mutation and glucose on alternative splicing of the *SR45* and At2g33830 pre-mRNAs.

Schematic diagrams of two splice variants (SVs) produced by the *SR45* **(A)** and At2g33830 **(B)** genes (boxes represent exons with UTRs in black, lines represent introns, while arrowheads indicate alternative 3' splice sites), and histograms showing the proportion of each transcript in wild-type (Col-0) and mutant (*sr45-1*) seedlings grown under control or glucose conditions.

4.3.5. The splicing pattern of a dormancy/auxin-associated gene is dramatically altered in the *sr45-1* mutant

The alternative splicing RT-PCR panel includes primer pairs specific to the genes encoding the SnRK1.1 and SnRK1.2 protein kinases. In agreement with our analysis of the splicing pattern of the *SnRK1.1* gene (see Figure 4.2A), no significant changes were observed in the relative abundance of two alternative *SnRK1.1*-specific transcripts analyzed by the panel. However, the ratio of *SnRK1.2* alternatively-spliced forms was changed by about 15% in the *sr45-1* mutant (Table B in Appendix I), with the mutation increasing the selection of a distal 5' splice site in the gene's first intron and thereby the abundance of a transcript carrying a longer 5' untranslated region (UTR). Although glucose had a similar, albeit less striking, effect on this alternative splicing event (Table C in Appendix I), no further changes in the *SnRK1.2* splicing pattern were observed when the mutant was grown in the presence of glucose.

We then turned to identify the genes showing the greatest change in alternative splicing as a result of the *sr45-1* mutation. As seen in Table 4.1, among the 93 genes whose splicing pattern was altered by the mutation, only eight exhibited changes in the ratio of alternative splice variants of at least 20%. These include genes encoding DNA- and RNA-binding proteins, factors involved in protein-protein interactions, a chloroplast triose-phosphate transporter, and proteins with unknown molecular function. Consistent with an earlier report (Ali et al., 2007), our results indicate that the alternative splicing pattern of the SR protein gene *RS31* is markedly changed in the *sr45-1* mutant. Table 4.2 shows that glucose exerted a less dramatic effect on alternative splicing than the *sr45-1* mutation. Only four genes — encoding three transcription factors and a MAP kinase — had their splicing patterns changed by 20% or more in the wild type, whereas applying the same stringent criteria only one — At2g33830 — was changed by the sugar in mutant plants (Table 4.2). Interestingly, the latter was also the gene undergoing the most dramatic variation in alternatively-spliced transcripts as a result of the *sr45-1* mutation (Table 4.1).

Table 4.1. Genes showing substantial alternative splicing pattern changes in the *sr45-1* mutant.

Locus ID	Gene description	AS type	Primer pair	Product size (bp)	Variation (%)	*P*-value
At2g33830	Auxin-regulated protein (dormancy)	3'SS	243	140	+ 52.33	0.000
				147	- 52.33	0.001
At4g12790	Unknown protein	3'SS	36	212	- 28.50	0.000
				341	+ 32.82	0.000
At2g34830	WRKY family transcription factor	3'SS	126	199	- 24.82	0.002
				217	+ 25.20	0.001
At4g27050	F-box family protein	Unknown	155	178	- 16.60	0.000
		Unknown		233	- 5.66	0.000
				352	+ 23.20	0.000
At5g46110	P/trioseP Translocator precursor	Unknown	245	124	- 19.97	0.016
				130	+ 22.40	0.009
At1g15200	atPinin		224	130	+ 18.21	0.000
		Unknown		236	- 22.21	0.000
		3'SS		313	- 5.39	0.000
At3g61860	SR protein — RS31		205	158	+ 19.41	0.000
		3'SS		670	- 21.00	0.000
At2g16940	RNA recognition motif (RRM)-containing protein		209	136	+ 18.14	0.000
		3'SS		418	- 20.63	0.000

The genes presented showed a ≥ 20% variation in the abundance of at least one of their alternatively-spliced transcripts when *sr45-1* mutant plants were compared with the wild type under control conditions. For each gene, only the RT-PCR products displaying significant changes (> 3%; *P* < 0.05) in pairwise comparisons between biological samples are shown. AS, alternative splicing; 3'SS, alternative 3' splice site.

Notably, of the genes included in the panel, At2g33830, encoding a dormancy/auxin-associated family protein with unknown function, displayed by far the most pronounced changes in the ratio of its alternative splice variants. As shown in Figure 4.6B, this gene expresses almost exclusively (> 98% of total transcript levels) one mRNA in wild-type seedlings grown both under control and glucose conditions, but loss of SR45 function strikingly increased selection of a distal 3' splice site in the gene's first intron, resulting in a sharp increase in the abundance of a 6-nucleotide shorter transcript (Figure 4.6B). In fact, the proportion of the two alternative splice forms changed from 99%:1% to 46%:54% as a result of the *sr45-1* mutation (Table B in Appendix I). Remarkably, and despite having no effect on processing of the At2g33830 mRNA in the wild-type, the presence of glucose was able to almost

completely restore the original splice variant ratio in the mutant (Figure 4.6B), thus compensating for the absence of the SR45 splicing factor. This SR45 molecular target may therefore contritute to pleiotropic developmental phenotypes of the *sr45-1* mutant, but is unlikely to determine its defect in sugar signaling.

Table 4.2. Genes whose alternative splicing pattern is substantially changed by the presence of glucose in the wild type or the *sr45-1* mutant.

Locus ID	Gene description	AS type	Primer pair	Product size (bp)	Variation (%)	P-value
Genes whose AS is changed by glucose in the wild type						
At1g09000	NPK1-related protein kinase, putative (ANP1); OXIDATIVE; COLD	Unknown	358	189	- 14.04	0.013
				316	+ 24.68	0.017
At2g34830	WRKY family transcription factor	3'SS	126	199	- 24.71	0.002
				217	+ 23.37	0.001
At4g34430	DNA-binding family protein	3'SS	156	192	- 20.77	0.001
				197	+ 20.77	0.001
At5g02470	DP-2 transcription factor, putative (DPA)		187	165	- 21.39	0.001
		5'SS		293	+ 21.39	0.003
Genes whose AS is changed by glucose in the sr45-1 *mutant*						
At2g33830	Auxin regulated protein (Dormancy)	3'SS	243	140	- 39.03	0.004
				147	+ 39.03	0.003

The genes presented showed a ≥ 20% variation in the abundance of at least one of their alternatively-spliced transcripts when wild-type or *sr45-1* plants grown in the presence of 3% glucose were compared, respectively, with wild-type or *sr45-1* plants grown under control conditions. For each gene, only the RT-PCR products displaying significant changes (> 3%; $P < 0.05$) in pairwise comparisons between biological samples are shown. AS, alternative splicing; 3'SS, alternative 3' splice site; 5'SS, alternative 5' splice site.

4.4. Discussion

We have previously shown that the Arabidopsis *sr45-1* mutant, initially described to display pleiotropic developmental defects under normal growth conditions (Ali et al., 2007), is impaired in glucose and ABA signaling during early seedling development (Carvalho et al., 2010). In the present report, we demonstrate that the SR45 RNA-binding protein controls glucose responses via a mechanism independent of HXK1, an

evolutionarily conserved sugar sensor that integrates nutrient and hormone signals to regulate gene expression and plant growth and development (Moore et al., 2003). Our results indicate that steady-state *HXK1* transcript levels are unaffected by loss of function of the SR45 protein and, most importantly, that the glucose hypersensitivity of the *sr45-1* mutant is retained in the absence of *HXK1* expression, clearly showing that the proteins encoded by these two genes are operating in distinct pathways.

The finding that the SR45 splicing factor acts via a HXK1-independent glucose signaling mechanism turned our attention to the SnRK1 subfamily of plant protein kinases that, as their orthologs SNF1 in yeast and AMPK in mammals, appear to act as master metabolism regulators. Importantly, we show here that upon glucose treatment the *sr45-1* mutant contains slightly but significantly enhanced amounts of SnRK1 protein compared to the wild type. SnRK1s have been demonstrated to play a pivotal role under sugar and energy deprivation conditions (Baena-Gonzalez et al., 2007), and transgenic Arabidopsis plants overexpressing SnRK1.1 are hypersensitive to sugars during early seedling development (Baena-Gonzalez et al., 2007; Jossier et al., 2009). In addition to enhanced glucose sensitivity, the *sr45-1* mutant shows hypersensitivity to ABA, with our previous work providing evidence that the SR45 splicing factor negatively regulates glucose signaling by repressing the ABA pathway (Carvalho et al., 2010). Interestingly, SnRK1.1 overexpression in Arabidopsis also results in ABA hypersensitivity, suggesting a role for this kinase in linking the sugar and ABA signal transduction pathways (Jossier et al., 2009). Unlike the *sr45-1* mutant, which shows enhanced ability to accumulate ABA in response to glucose (Carvalho et al., 2010), these *35S:SnRK1.1* transgenic lines are reportedly unaffected in their ABA content (Jossier et al., 2009). However, decreased ABA levels have been found in SnRK1-deficient pea (*Pisum sativum*) antisense lines (Radchuk et al., 2010), which exhibit an ABA-insensitive-like phenotype (Radchuk et al., 2006). In Arabidopsis, the enhanced glucose and ABA sensitivity displayed by both the *sr45-1* mutant and SnRK1.1 overexpressor plants is observed as a postgermination growth arrest, which functions as a developmental checkpoint to monitor adverse environmental conditions in the transition from hetero- to autotrophic growth (Lopez-Molina et al., 2001). Jossier

et al. (2009) have proposed that the SnRK1.1 protein kinase may contribute to regulate ABA-sugar interactions during this transition.

Our results also indicate that the enhanced SnRK1.1 levels observed in the *sr45-1* mutant are a result of reduced proteasomal degradation of the protein kinase. SnRK1.1 activity under low nutrients or high glucose conditions has been shown to require the presence of an Arabidopsis myoinositol polyphosphate 5-phosphatase, 5PTase13, which reduces the amounts of SnRK1.1 protein targeted to the proteasome (Ananieva et al., 2008). Consistent with the 5PTase13 protein positively regulating SnRK1.1 at high glucose concentrations, mutations in the *5PTase13* locus lead to the opposite phenotype conferred by gain of SnRK1.1 and loss of SR45 function — insensitivity to both sugar and ABA during early seedling development. A key finding of the present study is that the *5PTase13* pre-mRNA is a splicing target of the SR45 protein, which is required for efficient processing of the gene's sixth intron. Given the described role of this inositol phosphatase in stabilizing the SnRK1.1 kinase (Ananieva et al., 2008), we propose that SR45 regulates SnRK1.1 levels in response to sugars by modulating alternative splicing of the *5PTase13* gene. Our results suggest that without changes in total gene expression levels, a shift in the *5PTase13* splicing pattern from the fully spliced variant to a longer transcript containing a retained intron leads to stabilization of the SnRK1.1 protein. Future functional analysis of the individual *5PTase13* splice variants should conclusively establish the in vivo role of each isoform in the regulation of SnRK1.1 stability and validate the *5PTase13* mRNA as a functional target of the SR45 splicing factor.

To gain further insight into the mode of action of SR45, we widened our search for its mRNA targets taking advantage of a high-resolution panel to examine multiple Arabidopsis alternative splicing events simultaneously (Simpson et al., 2008). About 90 and 80 genes of the 262 on the panel had their splicing pattern significantly altered by loss of SR45 function and glucose, respectively, with the effect of the mutation on alternative splicing being more pronounced than that of the sugar. Globally, the results

obtained using this tool do not support any clear bias for a particular gene functional category or type of alternative splicing event.

The RT-PCR panel does not include primers for the *5PTase13* gene, but the results obtained for the *SnRK1.1* gene verified the absence of alternative splicing changes in the *sr45-1* mutant. Our results also confirm the previous finding that SR45 targets splicing of SR protein genes (Ali et al., 2007). In addition to marked changes in the ratio of alternative transcripts produced by the *RS31* gene, we found significant *sr45-1*-induced alterations in the splicing pattern of another four SR genes — *RS2Z32*, *SR34b*, *SCL30a* and *RS40* — either under control or glucose conditions. Moreover, the presence of glucose alone affected alternative splicing of six of the 11 SR protein genes included in the panel — *SR34*, *SR34a*, *SR34b*, *SCL33*, *RS2Z33* and *RS31*. Importantly, because the *sr45-1* mutant expresses truncated transcripts upstream of the T-DNA insertion site, we were able to show that the SR45 splicing factor regulates the selection of alternative 3' splice sites in the sixth intron of its own pre-mRNA. Autoregulation of SR and other splicing-related proteins has been reported in many systems, including for the Arabidopsis SR30 (Lopato et al., 1999) and RS2Z33 (Kalyna et al., 2003).

It is interesting to note that the SR45 protein significantly affects an alternative splicing event controlling the length of the 5' UTR in the *SnRK1.2* mRNA. Given the established role of this region in regulating transcript stability or translation, the higher fraction of the longer 5' UTR splice variant found in the *sr45-1* mutant could explain its enhanced SnRK1.2 protein levels. On the other hand, the gene whose splicing pattern is most strikingly changed by the *sr45-1* mutation encodes two predicted isoforms of an uncharacterized dormancy/auxin-associated family protein that differ in only two amino acids. While the shorter splice form is barely expressed in the wild type, it accounts for more than 50% of total transcript levels in the mutant. Future functional analysis of these and other individual splice isoforms shown here to be under SR45 control should elucidate the physiological relevance of the splicing targets identified in this study, while disclosing the precise molecular mechanisms underlying the role of the SR45 protein in plant development and sugar signaling. Furthermore, RNA-

immunoprecipitation assays may clarify whether SR45 regulates alternative splicing of its multiple targets directly, or indirectly potentially via changes in the proportion of the splice isoforms of other splicing regulators such as SR proteins.

4.5. Materials and methods

4.5.1. Plant materials and growth conditions

The *Arabidopsis thaliana* Colombia (Col-0) ecotype was used as the wild type in all experiments. A homozygous line for the *sr45-1* T-DNA insertion mutant (SALK_004132) was previously isolated by Carvalho *et al.* (2010), and seeds homozygous for T-DNA insertions within the *HXK1* locus, *hxk1-1* (SALK_018086) and *hxk1-2* (SALK_070739), were kindly provided by Dr. S. Yanagisawa (University of Tokyo, Japan). To generate *sr45-1 hxk1* double mutants, the *sr45-1* mutant was crossed with the *hxk1-1* or *hxk1-2* alleles, and F_2 plants homozygous for T-DNA insertions in the both the *HXK1* and the *SR45* loci were selected by PCR genotyping using *HXK1-* and *SR45*-specific primers (Table 4.3) as well as primers annealing at the left border of the T-DNA.

Seeds were surface-sterilized and sown in petri dishes containing Murashige and Skoog (MS) salts (Duchefa Biochemie), 2.5 mM MES (pH 5.7), 0.5 mM myoinositol, and 0.8% agar. After stratification for 3 days in the dark at 4ºC (to break dormancy), the seeds were transferred to a growth chamber under 16 h photoperiod (90 mmol m^{-2} s^{-1} white light) at 22ºC and 60% relative humidity. After 2-3 weeks, plants were transferred to soil in individual pots.

Table 4.3. Sequences of the gene-specific primers used in RT-PCR analyses.

Name	Gene ID	Forward primer	# cycles
HXK1	At4g29130	Fwd: 5'-GTTGGAGCGACTGTTGTTTG-3'	26
		Rev: 5'-ACTCTCGAAATCCAGCGTGT-3'	
SR45	At1g16610	Fwd: 5'-AACGTTCACACTACCACCTCG-3'	26
		Rev: 5'-GTAAGAAGATGACCTCCCACG-3'	
KIN10	At3g01090	F1: 5'-TGCATTTTTTGGTTCCGAATTTTC-3'	30
		R1: 5'-CATCTCCATGTTCTTGATTTTG-3'	
		F2: 5'-CAATGGATTGATCTTGAAATC-3'	30
		R2: 5'-ATAGAGACGGATGATGTGAGG-3'	
DIN1	At4g35770	Fwd: 5'-CAGAGTCGGATCAGGAATGG-3'	31
		Rev: 5'- GGTTGTGAGAGCGGTCAAAT-3'	
SEN5	At3g15450	Fwd: 5'-GCGAAACTCTCTCCGACTTC-3'	30
		Rev: 5'-CCACAGAACAACCTTTGACG-3'	
DIN6	At3g47340	Fwd: 5'-AGGTGCGGACGAGATCTTTGG-3'	33
		Rev: 5'-GTTGTCAATTGCCTTAAGTGG-3'	
5PTase13	At1g05630	F3: 5'-AGAAACGGTTGGACTTGAAGG-3'	33
		R3: 5'-ATAAGCTGAAGTTGAGACACC-3'	
ACT2	At3g18780	Fwd: 5'-TTTGCAGGAGATGATGCTCCC-3'	25
		Rev: 5'- GTCTTTGAGGTTTCCATCTCC-3'	

4.5.2. Growth and glucose assays

Seeds were surface-sterilized and water-imbibed in the dark for 3 days at 4ºC. After stratification, 80 to 100 seeds of each genotype were sown in triplicate in petri dishes containing MS medium (MS salts, 2.5 mM MES, pH 5.7, 0.5 mM myoinositol, and 0.8% agar), supplemented or not with the appropriate concentrations of D-glucose, before transfer to the growth chamber (16-h photoperiod). After 7 days, cotyledon greening rates were calculated over the total of germinated seeds. For assessment of hypocotyl elongation, 20 to 50 seeds of each genotype were surface-sterilized and stratified as described above, plated in MS plates supplemented or not with the appropriate concentrations of D-glucose and grown vertically in complete darkness at 22ºC for 7 days. Etiolated seedlings were illuminated for 12-h before hypocotyl length measurements using the NIH ImageJ software (http://rsbweb.nih.gov/ij).

For glucose transient assays in leaves, seeds were directly sown in soil after stratification, and pots placed in a growth chamber under a 12 h photoperiod (100

mmol m^{-2} s^{-1} white light) at 22ºC and 60% relative humidity. Leaf disks from 5/6-week old plants were immersed in water (control conditions), 1.5% D-glucose or 1.5% D-sorbitol. The plant material was collected after 6 h and frozen at -80ºC until RNA or protein extraction. For inhibition of protein synthesis or proteasomal degradation, 100 mM cycloheximide (SIGMA) or 50 mM MG132 (Calbiochem) were added 1 h prior to the onset of the glucose treatment.

4.5.3. RNA extraction and RT-PCR analyses

Total RNA was extracted from whole Arabidopsis seedlings using the innuPREP Plant RNA kit (Analytik Jena BioSolutions) and from leaves using TriReagent (Sigma), following the manufacturers' protocols. All RNA samples were digested with DNaseI (Promega) and phenol-chloroform purified before reverse transcription with M-MLV reverse transcriptase (Promega). The primers used are listed in Table 4.3. Preliminary PCRs were carried out with different cycles to determine the linear range of amplification. Based on these analyses, 20 to 35 cycles were chosen for DNA detection, depending on the gene (Table 4.3). Amplified DNA fragments were separated in 1% agarose gels. Each RT-PCR experiment was repeated independently at least three times to verify the observed changes in expression. For the *5PTase13* gene, band intensities were quantified using the NIH ImageJ software (http://rsbweb.nih.gov/ij) and transcript levels normalized to *actin2* before calculating the ratio between the two splice variants.

4.5.4. Protein extraction and western blot analyses

For total protein extraction, frozen Arabidopsis leaves were ground with a mortar and pestle in extraction buffer containing 50 mM Hepes/NaOH (pH 7.5), 10 mM MgCl$_2$, 1 mM EDTA, 2 mM dithiothreitol and one tablet of the Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science). The homogenates were centrifuged at maximum speed (4ºC) and the protein content of the supernatants determined spectrophotometrically at 595 nm using a protein assay kit based on the Bradford

method (Bio-Rad), using bovine serum albumin as a standard. Equal amounts of protein were then resolved under reducing conditions in 10% SDS/polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore), which were incubated with SnRK1.1 or SnRK1.2 primary antibodies (kindly provided by Dr. E. Baena-González; diluted 1:4,000) overnight at 4ºC, and then with anti-rabbit peroxidase-conjugated secondary antibodies (Amersham Pharmacia; diluted 1:20,000) for 2 h at room temperature, in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl) supplemented with 1% nonfat dry milk. After washing the membranes for 30 minutes with TBS containing 0.05% Tween 20, membrane-associated peroxidase activity was visualized by ECL. Western blots were scanned and band intensities quantified using the software NIH IMAGEJ program (http://rsb.info.nih.gov/ij), with protein levels being normalized to the RuBisCO large subunit band visualized in Ponceau-stained membranes.

4.5.5. Alternative splicing high-resolution RT-PCR panel

Wild-type (Col-0) and mutant (*sr45-1*) seedlings were grown in MS plates supplemented with or without 3% glucose and the plant material (100 mg) harvested at the same developmental stage (approximately 50% cotyledon expansion). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and quantified spectrophotometrically at 260nm. RT-PCR experiments were performed on total RNA after DNase I (RNase-free DNase set, Qiagen) treatment, according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 5 mg of RNA using "Ready-to-go you-prime" first strand beads (GE-Healthcare) and 2 mL of oligo d(T)^{18} (Invitrogen). The reverse-transcription reaction was diluted to a final volume of 100 mL, and 1 mL was aliquoted into a 96-well reaction plate along with 1X PCR buffer with MgCl$_2$ (Roche), 200 mM of each dNTP (Promega), 1.5 mM of each of the alternative splicing event-specific primers and Taq DNA polymerase (Roche) with a standard PCR reaction being performed at 24 cycles. This number of PCR cycles was previously shown to be in the linear range of amplification for mRNA transcripts of numerous genes (Simpson et al., 2008). Alternative splicing event-specific primers

were selected to amplify the expected alternatively-spliced transcripts and yield RT-PCR products of sizes between 70 and 700 bp. In order to visualize the RT-PCR products, the forward primer was labeled with 6-Carboxyfluorescein (6-FAM) dye. Primer sequences are given in Table E in Appendix I.

Labeled RT-PCR product (1 mL) from the RT-PCR reactions was mixed with 8.95 mL Hi Di Formamide (Applied Biosystems) and with 0.05 mL of GeneScan 500 LIZ internal size standard (Applied Biosystems). Using an ABI 3730 DNA Analyzer (Applied Biosystems), capillary electrophoresis of the RT-PCR fragments was performed. Product size and peak area data were determined by *Genemapper* software analysis (Applied Biosystems). RT-PCR products were accurately identified with ± 1-nt resolution. The relative fluorescent peak areas for RT-PCR products with expected sizes for the alternatively-spliced products were extracted, and the ratio of the alternatively-spliced products was calculated by dividing the value of each spliced product by the sum of the values of all products. A minimum of three biological repetitions was performed to determine statistically significant changes in alternative splicing under the conditions tested. The mean alternative splicing ratios with standard errors were calculated for the three separate biological repetitions. Pairwise comparisons were made between wild type and mutant plants, grown under control and glucose conditions. Analysis of variance was used to determine significant variation between the means. Alternative splicing events with significant variation ($P <$ 0.05) were selected.

The alternative splicing RT-PCR panel includes 262 genes predicted to undergo alternative splicing and two controls (Table A in Appendix I). Genes were selected from annotated events found in different databases — ASIP (http://www.plantgdb.org/ASIP/EnterDB.php), TIGR (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=arab), RIKEN (http://rarge.gsc.riken.jp/) and TAIR (http://www.arabidopsis.org/) — and encoded mostly transcription factors, RNA binding proteins, and stress-related genes. The primer pairs specific for the two control genes

amplified either constitutively spliced introns (At3g12110) or a U12-dependent intron (At4g02560).

R. Carvalho was the main responsible for the experimental work and data analysis.

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CHAPTER 5

Concluding remarks and future perspectives

Plants posses a high level of developmental plasticity and stress tolerance, which compensates for their lack of mobility. The capacity to reprogram their whole transcriptome by switching on specific genes while switching off others allows these organisms to respond to various environmental challenges. Alternative splicing provides a quick and adjustable means of gene regulation and is likely to be important in plant stress responses. In fact, genome-wide analyses have revealed that alternative splicing is highly widespread in plants, and genes involved in stress responses appear overrepresented in gene ontology analyses of alternatively-spliced genes (Iida et al., 2004; Wang and Brendel, 2006; Filichkin et al., 2010; reviewed in Ali and Reddy, 2008 and Duque, 2011). SR proteins constitute an important family of RNA-binding proteins that function in the regulation of both constitutive and alternative splicing. These proteins are evolutionarily conserved in organisms undergoing alternative splicing and have been extensively studied in animals but to a lesser extent in plants (Reddy, 2007; Long and Caceres, 2009; Simpson et al., 2010; Reddy and Shad Ali, 2011). The members of this family of splicing factors act as key factors in the early recognition of splice sites and, as master regulators of alternative splicing, are able to influence splice site selection under different conditions in a concentration- and phosphorylation-dependent manner.

The overall goal of the present thesis was to contribute to the characterization of Arabidopsis SR-related proteins in an attempt to uncover their potential roles in plant stress responses. In order to address this matter, it was important to first assess the stress regulation of these splicing factors at the transcriptional level, which could represent an essential basis for the in vivo functional analysis of each individual gene. Finally, the identification of physiological transcripts targeted by these master regulators of pre-mRNA splicing would provide important clues in unraveling the precise molecular mechanisms underlying their specific roles in plant development and stress responses.

The work reported here started out by analyzing the tissue- and stress-specific expression and splicing patterns of the family of Arabidopsis SR protein genes as well as of the SR45 non-canonical SR protein (Chapter 2). When this study was initiated, the only existent reports on the expression of individual SR genes involved northern blot analyses in a very limited range of vegetative tissues, and none addressed the transcriptional behavior of these genes under different stress conditions. Our detailed analysis of Arabidopsis SR-related genes shows that the majority of them are expressed in both embryonic and vegetative tissues. With a few exceptions, in embryonic tissues all members of this important class of splicing factors are barely detected in seeds but their expression is activated upon imbibition, suggesting they could play a role in seed germination. In vegetative tissues, both SR genes and *SR45* show highest gene expression levels in flowers. Furthermore, five genes, including *SR45*, undergo alternative splicing. While this work was being developed, an overall alternative splicing analysis of Arabidopsis SR-related protein genes was published by Palusa et al. (2007). These authors investigated the expression and splicing patterns of the SR gene family and of the *SR45* gene in different vegetative tissues and in seedlings of different ages, as well as in plants submitted to the effect of phytohormones and environmental stress. Likewise, we had begun a similar analysis with abscisic acid (ABA) treatment and exposure to cold stress and, for the *SR45* gene, added glucose treatment as well as exposure to other abiotic stresses such as high salinity, heat and drought. These experiments were not extended to the 18 SR protein genes as they had just been reported by Palusa et al. (2007). Our results show that expression of the vast majority of SR genes is affected by both cold and ABA treatments, with the splicing pattern of two SR genes being altered by cold. The *SR45* gene is affected by ABA, salt and temperature stress. Such results support the notion that rapid reprogramming of the transcriptome in response to external signals may occur through regulation of the expression of SR-related genes.

Despite a few discrepancies, our analyses along with those of Palusa et al. (2007) present data that contribute to a global picture of the regulation and tissue-specificity of

SR-related gene expression. Together, these results will provide invaluable clues in determining the exact function of each member of the Arabidopsis SR protein family as well as of SR45. The latter, although recently excluded from the SR gene family (Barta et al., 2010), was demonstrated by Ali et al. (2007) to function as an essential splicing factor owing to its ability to complement an animal splicing-deficient extract.

Since the mid 1990s, multiple research groups have identified and contributed to the characterization of Arabidopsis SR proteins (Lazar et al., 1995; Lopato et al., 1996a; Lopato et al., 1996b; Golovkin and Reddy, 1999; Lopato et al., 1999a; Lopato et al., 1999b; Lazar and Goodman, 2000; Lopato et al., 2002; Ali et al., 2003; Fang et al., 2004). However, no loss-of-function mutants for any of the 18 SR proteins have been described. Instead, proposed functions are based on the overexpression of SR proteins in wild-type Arabidopsis plants — overexpression of either At*SR30* and At*RS2Z33* has been found to lead to severe developmental abnormalities in both embryos and young plants (Lopato et al., 1999a; Kalyna et al., 2003). Importantly, the present study reports the isolation of four SR protein genetic null mutants — *sr34, scl30a, scl33* and *rs31*. Future phenotypical, biochemical and molecular characterization of these mutants will provide pivotal resource information on the biological roles of plant SR proteins. The pre-mRNAs of Arabidopsis SR protein genes often undergo alternative splicing themselves, and have been reported to encode more than 50 putative proteins, many of which lack one or more domains (Palusa et al., 2007; Reddy and Shad Ali, 2011). These truncated versions of SR proteins may be nonfunctional or have altered functions. Expressing each splice variant of a given SR gene in its knockout background will allow assessment of the biological significance of each individual SR protein isoform. SR gene loss-of-function mutants will also be indispensable in the identification of genes whose transcripts are targeted by SR proteins, which will in turn provide invaluable functional clues in the characterization of each SR protein.

In addition to the four SR protein mutants mentioned above, the isolation of a knockout mutant for the non-canonical SR gene *SR45* is also described in the present

work. This same mutant, named *sr45-1*, was isolated in parallel and described in 2007 by Reddy and co-workers (Ali et al., 2007), providing important information on the in vivo functions of SR45 — it is implicated in several aspects of plant development such as root growth, flowering, leaf and flower morphology, and overall plant size. However, the potential role of SR45 in plant stress responses had remained unaddressed.

The remaining work presented in this thesis focused on the further characterization of the SR45 splicing factor. A first study (Chapter 3) specifically addressed the involvement of this non-canonical SR protein in plant responses to external cues. Other than the developmental defects previously described by Ali et al. (2007), the *sr45-1* mutant displays no additional phenotypes when grown under high salinity, drought, cold or heat stress conditions. However, this loss-of-function mutant clearly shows altered responses to both glucose and the plant stress hormone ABA. Our results indicate that the mutant's hypersensitivity to glucose is a result of increased ABA signaling gene expression and enhanced ability to accumulate the phytohormone in response to glucose. We therefore define the Arabidopsis SR45 as negatively regulating sugar signaling through downregulation of the ABA pathway during early seedling development.

Two protein isoforms of SR45 arising from alternative splicing of the *SR45* pre-mRNA have been shown to complement different *sr45-1* developmental phenotypes observed under normal growth conditions (Zhang and Mount, 2009). However, we found that the alternatively-spliced region of SR45 does not play a role in glucose signaling during early seedling growth, indicating that while these alternative splice variants display specificity when affecting some processes, they may play a general role in others.

A follow-up study (Chapter 4) began by investigating the role of a conserved glucose sensor, hexokinase1 (HXK1), in the mode of action of SR45. In light of the *sr45-1* glucose hypersensitivity, we investigated whether SR45 is acting via an HXK-

mediated or HXK-independent pathway. As it happens, disruption of the *HXK1* gene does not suppress the glucose phenotypes of the *sr45-1* mutant, clearly showing a mechanism independent of this sugar sensor. We then examined the potential role of Arabidopsis SnRK1 protein kinases, which have been shown to perform similar tasks to their homologous counterparts in yeast (SNF1) and mammals (AMPK) (Baena-Gonzalez et al., 2007) — turning off energy-consuming anabolic pathways, while turning on energy-producing catabolic processes in response to a sugar/energy deprivation signal. In addition, SnRK1.1 has been described as a central player in both sugar and ABA signaling pathways. In fact, Jossier et al. (2009) reported that transgenic plants overexpressing SnRK1.1 also display hypersensitive responses to glucose and ABA. Notably, glucose-treated *sr45-1* leaves contain significantly higher amounts of the SnRK1.1 kinase due to lower degradation rates of the protein in the mutant. An inositol polyphosphate 5-phosphatase, 5PTase13, has been previously shown to interact with and regulate the stability of SnRK1.1 in vitro (Ananieva et al., 2008). Importantly, the present work shows that the *sr45-1* mutation markedly alters the ratio of the two alternatively-spliced transcripts produced by the *5PTase13* gene, providing a mechanistic link between SR45 function and the modulation of SnRK1.1 levels in response to sugars.

Arabidopsis SR proteins have been found to affect alternative splicing of their own pre-mRNAs as well as of those encoded by several other SR protein genes (Lopato et al., 1999a; Kalyna et al., 2003; Isshiki et al., 2006). Prior to this study, the only genes known to have their splicing patterns affected by SR45 were five SR genes (Ali et al., 2007). The identification of the physiological targets of this splicing factor is crucial to fully understand its role in both plant development and the response to abiotic stress. Genes that undergo alternative splicing may be regulated in a very specific manner with very precise environmental and tissue-specific requirements, rendering it difficult to pinpoint the transcripts targeted by a given splicing factor. A very recent study has reported the use of tiling arrays in Arabidopsis as an efficient tool for the global analysis of alternative splicing events (Yoshimura et al., 2011). Nevertheless, next-

generation deep-sequencing of transcriptomes holds the most promise in providing a detailed global analysis of the gene expression and splicing patterns affected by Arabidopsis SR-related genes.

In 2008, Simpson et al. reported the development of a high-resolution RT-PCR system to monitor changes in more than 300 Arabidopsis alternative splicing events. In the identification of additional SR45 splicing targets, this RT-PCR panel now allowed comparison of 498 splicing events in 242 genes between wild-type and *sr45-1* seedlings grown under control or glucose conditions. Interestingly, consistent with previous reports of autoregulation of the Arabidopsis SR proteins SR30 (Lopato et al., 1999) and RSZ2Z33 (Kalyna et al., 2003), we found that SR45 regulates its own splicing, favoring expression of the splice variant encoding its full-length protein. Moreover, the SnRK1.2 gene shows a slight but significant *sr45-1*-induced change in its splicing pattern, which may have consequences on the stability or translation of its mRNA. Although unlikely to be involved in the sugar signaling defect caused by loss of SR45 function, the gene showing the most dramatic change in its splicing pattern as a result of the *sr45-1* mutation is At2g33830, which encodes a dormancy/auxin-associated family protein with unknown function. Future validation of these and other transcripts identified using this alternative splicing tool as functional targets of the SR45 splicing factor will be essential in uncovering the precise mechanisms governing its role in plant development and sugar signaling.

Identifying the physiological targets determining the mode of action of the SR45 protein is indeed crucial. In order to investigate whether the alteration in splicing observed for a given target transcript is functionally relevant, it will be necessary to evaluate whether restoring the target's original splicing pattern enables complementation of the developmental and stress phenotypes conferred by the loss of function of SR45. To this end, the functional significance of individual splice isoforms shown to be under control of the SR45 protein should be analyzed in the knockout background under the appropriate growth conditions. This may allow confirmation of

the physiological relevance of the splicing target in plant development and stress tolerance, while revealing the molecular mechanisms governing the functions of the SR45 protein in these processes.

Another important question is whether the targets identified in Chapter 4 are being directly modulated by SR45 or whether the observed alternative splicing changes are a consequence of other downstream events controlled by SR45. In fact, the direct processing of these mRNAs could be carried out for example by the SR proteins whose genes also show altered splicing patterns in the *sr45-1* mutant. One way to address this issue is through RNA-immunoprecipitation assays. Isolating the mRNAs bound to the SR45 protein will allow RT-PCR verification of which gene transcripts identified so far represent direct SR45 targets. Furthermore, the combination of RNA immunoprecipitation with deep-sequencing approaches (RIP-seq) offers the possibility of genome-wide identification of the direct targets of RNA-binding proteins.

It will also be important to follow up on the interconnection between SR45 and the SnRK1.1 kinase. In addition to the 5PTase13 protein, another player in SnRK1.1 stability has been described. PRL1 was reported to act as the substrate receptor for the degradation of SnRK1.1 by a CUL4 (cullin4) E3 ligase in Arabidopsis (Lee et al., 2008). In accordance, the absence of the PRL1-CUL4 E3 ubiquitin ligase results in increased amounts of SnRK1.1 in the *prl1* and *cul4* mutants, which could account for the phenotypes displayed by these mutants such as their sugar and ABA hypersensitivity (Lee et al., 2008). We have verified that the *sr45-1* mutation does not affect the expression or splicing patterns of the *PRL1* gene. However, it may prove valuable to investigate the levels of the PRL1 protein in the *sr45-1* background. If indeed PRL1 mediates SnRK1.1 degradation, low levels of this protein in the *sr45-1* mutant could also contribute to the observed increased stability of the SnRK1.1 energy sensor in the absence of SR45.

Finally, another promising line of work would be to investigate the role of phosphorylation in the regulation of the SR45 protein. Similarly to their animal conterparts, plant SR proteins are known to be extensively phosphorylated at serine residues in their RS domains and several, including the non-canonical SR45, have been shown to be phosphorylated in vivo (de la Fuente van Bentem et al., 2006). One of the two characterized splice isoforms of SR45 lacks a putative phosphorylation site, which is responsible for directing each isoform to different biological functions (Zhang and Mount, 2009). The fact that there are many SR protein alternative splice forms that lack one or more phosphorylation sites suggests that phosphorylation could be an important posttranslational modification regulating the alternative splicing of SR protein target genes. Moreover, stress signals are known to control the phosphorylation status of plant SR proteins. In fact, a few protein kinases whose activity is regulated by various types of stress have been shown to phosphorylate several SR proteins (Savaldi-Goldstein et al., 2000; Feilner et al., 2005). These observations not only substantiate a link between stress and the regulation of splicing, but also underscore phosphorylation as a potential key mechanism for relaying stress signals to the splicing machinery. Future studies on the phosphorylation dynamics of SR-related splicing factors such as the SR45 protein may therefore open new avenues for a basic understanding of the role of alternative splicing in plant responses to abiotic stress.

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Appendix I

Table A. Genes and alternative splicing events included in the RT-PCR panel.

Locus ID	Gene description	Primer pair	Products (bp)	AS type
At1g01060	LHY late elongated hypocotyl - Myb-like DNA binding	305	**161**	
			232	Unknown
			331	Intron R
At1g01140	SOS2-like protein kinase PKS6/CBL-interacting protein kinase 9 (CIPK9);COLD	334	102	Unknown
			106	
			205	3'SS
At1g02090	COP9 signalosome complex subunit 7ii	241	**119**	3'SS
			138	
At1g02840	pre-mRNA splicing factor SF2 (SR1)	1	**63**	
			421	3'SS
			490	Unknown
			503	Unknown
At1g04400	Cryptochrome 2 apoprotein (CRY2) (PHH1)	2	**262**	
			329	3'SS
At1g04950	TATA box-binding protein-associated factor (TAF) family protein	236	134	Unknown
			152	5'SS
			267	
			282	Unknown
			295	Unknown
At1g07350	transformer serine/arginine-rich ribonucleoprotein	193	**200**	
			279	Exon S
			282	3'SS,5'SS
			294	3'SS
			297	Unknown
			443	3'SS
			452	5'SS
At1g07830	ribosomal protein L29 family protein	237	121	3'SS
			193	
			273	Unknown
At1g09000	NPK1-related protein kinase, putative (ANP1); OXIDATIVE; COLD	358	189	Unknown
			242	Intron R
			316	
At1g09140	SF2/ASF-like splicing modulator (SRp30)	3	101	3'SS
			445	
At1g09230	RNA recognition motif (RRM)-containing protein	208	120	3'SS
			128	
			139	3'SS
At1g09530	phytochrome interacting factor 3 (PIF3)	112	**230**	
			291	5'SS
At1g11650	RNA-binding protein 45 (RBP45)	203	133	Exon S
			532	
At1g15200	atPinin	224	**130**	
			217	Unknown
			225	Unknown
			236	Unknown
			313	3'SS
At1g16610	SR45	217	154	3'SS
			169	Unknown
			175	
At1g18660	zinc finger (C3HC4-type RING finger) family protein	116	**322**	
			338	3'SS
At1g23970	Unknown Protein (Auxin regulated)	68	175	5'SS
			190	

Gene	Description		Value	Type
At1g27370	squamosa promoter-binding protein-like 10 (SPL10)	102	**163**	
			191	3'SS
At1g27770	calcium-transporting ATPase 1, plasma membrane-type / Ca(2+)-ATPase isoform 1 (ACA1) / plastid envelope ATPase 1 (PEA1); ABA	363	**144**	
			365	Intron R
At1g30200	F-box family protein	108	248	3'SS
			259	
At1g30500	CCAAT-binding transcription factor	103	274	3'SS
			288	
At1G31500	endonuclease/exonuclease/phosphatase family protein	239	**119**	
			134	3'SS
At1g31600	2OG-Fe(II) oxygenase family protein	214	163	3'SS
			170	
			645	Unknown
		215	135	3'SS
			142	
At1g33060	no apical meristem (NAM) family protein	105	271	3'SS
			283	
At1g37150	Holocarboxylase synthetase 2 (HCS2)	251	106	3'SS
			135	
At1g44910	FF domain-containing protein / WW domain-containing protein; splicing factor-like	382	**216**	
			301	Intron R
At1g48960	universal stress protein (USP) family protein	232	**175**	
			209	3'SS
At1g49730	protein kinase family protein;COLD	345	**237**	
			274	Unknown
			342	Intron R
At1g49950	DNA-binding protein, putative	106	**193**	
			236	5'SS
At1g53650	RNA-binding protein	270	176	5'SS
			195	
At1g54080	UBP1A	256	184	Unknown
			247	3'SS
			259	
			582	Unknown
At1g54100	aldehyde dehydrogenase, putative / antiquitin, putative; COLD; SALT; DESSICATION;ABA	357	**245**	
			353	Intron R
At1g54360	TATA binding protein associated 6b (TAF6)	70	129	5'SS
			152	
At1g55310	SC35-like splicing factor (SCL33)	7	**199**	
			252	Unknown
			293	Unknown
			361	3'SS
			364	3'SS
At1g55870	CAF1 family ribonuclease	234	151	3'SS
			171	
At1g59750	auxin-responsive factor (ARF1)	107	171	Unknown
			177	3'SS
			186	
At1g60850	RNA pol subunit	242	**107**	
			118	3'SS
At1g61660	basic helix-loop-helix (bHLH) family protein	111	**177**	
			186	5'SS
At1g64625	bHLH transcription factor	376	111	Exon S
			135	Unknown
			141	Unknown
			155	Unknown
			185	

AGI code	Description	Length	Position	Type
At1g67210	zinc knuckle (CCHC-type) family protein	286	175	3'SS
			180	
At1g67960	S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A	296	**145**	5'SS
			167	
At1g69080	universal stress protein (USP) family protein	235	163	5'SS
			205	
At1g69250	nuclear transport factor 2 (NTF2) family protein / RNA recognition motif (RRM)-containing protein;COLD	326	**147**	
			246	Intron R
At1g71860	protein tyrosine phosphatase; COLD; SALT	367	274	3'SS
			284	
			288	3'SS
			363	Unknown
			375	Unknown
At1g72050	zinc finger (C2H2 type) family protein	110	**207**	
			380	3'SS, 5'SS
At1g72560	tRNA export mediator exportin-t	249	138	5'SS
			148	
			174	Unknown
At1g72650	myb family transcription factor	113	**183**	
			201	5'SS
At1g76460	RNA recognition motif (RRM)-containing protein	372	**259**	
			268	3'SS
			314	3'SS
			483	Intron R
At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	148	190	5'SS
			212	
At1g76580	Squamosa promoter binding protein-like 16 (SPL16);COLD	323	156	3'SS
			199	
At1g77080	MADS affecting flowering 1 (MAF1)	109	118	3'SS
			158	
At1g77740	putative phosphatidylinositol-4-phosphate-5-kinase; ABA	350	**282**	
			355	Intron R
At1g78290	protein kinase 1-like protein; SALT; OSMOTIC	368	168	Unknown
			197	
			549	Intron R
At1g79650	DNA repair protein RAD23	13	**156**	
			174	3'SS
			182	3'SS
At1g79880	La domain-containing protein	199	**131**	
			169	5'SS
		200	104	3'SS
			122	
At2g02390	glutathione S-transferase zeta 1	295	**181**	
			202	3'SS
			368	Unknown
At2g02570	atSPF30	278	**153**	
			221	3'SS
			310	3'SS
At2g02960	zinc finger (C3HC4-type RING finger) family protein	118	**197**	
			200	3'SS
			222	3'SS
			233	Unknown
			237	Unknown
			240	Unknown
			289	Unknown
At2g04790	Expressed protein	72	**167**	
			190	5'SS

Gene	Description	Length	Position	Type
At2g15530	zinc finger (C3HC4-type RING finger) family protein	128	223	5'SS
			242	
			363	Unknown
			381	Unknown
At2g15970	similar to cold acclimation protein WCOR413 (Triticum aestivum);COLD	341	239	Unknown
			322	
			326	Intron R
At2g16940	RNA recognition motif (RRM)-containing protein	209	**136**	
			144	Unknown
			209	3'SS, 5'SS
			227	Unknown
			418	3'SS
			445	Unknown
At2g18300	basic helix-loop-helix (bHLH) family protein	121	**222**	
			228	3'SS
At2g18960	plasma membrane proton ATPase (PMA); ABA	351	113	Unknown
			118	
			215	Intron R
At2g20180	basic helix-loop-helix (bHLH) family protein	293	152	3'SS
			189	
At2g21620	RD2 protein (Auxin regulated)	73	**199**	
			216	5'SS
At2g21660	glycine-rich RNA-binding protein (GRP7)	206	172	Unknown
			181	
			349	5'SS
			494	Unknown
At2g22670	indoleacetic acid-induced protein 8 (IAA8)	117	282	3'SS
			288	
At2g26150	HsfA2	393	**531**	
			565	3'SS, 5'SS
At2g27230	transcription factor-related	149	207	5'SS
			244	
At2g28550	AP2 transcription factor like protein;COLD	316	205	Unknown
			262	
			332	Intron R
		317	**266**	
			338	Intron R
			341	Unknown
At2g29210	splicing factor PWI domain-containing protein	223	**151**	
			202	3'SS
At2g30260	small nuclear ribonucleoprotein U2B	218	134	3'SS
			171	
At2g31370	bZIP transcription factor (POSF21)	132	**193**	
			204	5'SS
			187	Unknown
			213	Unknown
			233	Unknown
			236	Unknown
			239	Unknown
At2g32250	far-red impaired responsive protein, putative	133	178	3'SS
			184	
			307	Unknown
At2g32330	Unknown protein	19	202	3'SS
			273	
At2g33120	Putative Synaptobrevin (SAR1) NUP160;COLD	413	124	Unknown
			155	Unknown
			164	
			188	5'SS

At2g33480	putative NAM (no apical meristem)-like protein;COLD	322	321	3'SS
			394	Unknown
			399	
			488	Unknown
At2g33830	Auxin regulated protein (Dormancy)	243	140	3'SS
			147	
At2g34830	WRKY family transcription factor	126	199	3'SS
			217	
			272	Unknown
At2g36000	Expressed protein	75	151	5'SS
			255	
At2g36010	E2F transcription factor-3 (E2F3)	122	270	3'SS
			275	
At2g37060	CCAAT-box binding transcription factor, putative	124	140	3'SS
			230	
			251	3'SS
			255	3'SS
At2g37340	RSZ33	21	**121**	
			263	Intron R
			341	3'SS
			481	3'SS
At2g38170	calcium exchanger (CAX1); COLD; SALT	359	**259**	
			364	Intron R
			368	Unknown
At2g38185	zinc finger (C3HC4-type RING finger) family protein	130	172	3'SS
			195	
At2g38880	histone-like transcription factor (CBF/NF-Y) family protein	131	**312**	
			373	5'SS
At2g39730	Rubisco activase (Auxin regulated)	23	**247**	
			258	3'SS
At2g40830	zinc finger (C3HC4-type RING finger) family protein	129	**218**	
			230	5'SS
			329	Unknown
			363	Unknown
At2g41710	ovule development protein, putative	120	230	3'SS
			245	
At2g42245	RNA binding protein-related	267	165	3'SS
			174	
			264	3'SS
At2g42890	RNA recognition motif (RRM)-containing protein	216	**147**	
			176	3'SS
At2g43010	phytochrome-interacting factor 4 (PIF4)	127	213	5'SS
			219	
At2g43410	FPA	314	**406**	
			540	Intron R
At2g43640	signal recognition particle 14 kDa family protein / SRP14 family protein	383	143	Unknown
			169	5'SS
			215	
At2g43810	putative small nuclear ribonucleoprotein polypeptide F	385	160	Unknown
			167	Unknown
			177	Exon S
			256	
			264	Unknown
At2g46270	G-box binding factor 3 (GBF3)	291	154	5'SS
			222	
At2g46370	auxin-responsive GH3 family protein	192	**200**	
			284	5'SS

At2g46790	timing of CAB expression 1-like protein (TL1)	125	**243**	
			251	5'SS
At2g46830	CCA1 Myb-like DNA binding	300	**218**	5'SS
			525	5'SS
At2g47580	small nuclear ...eristems... proteins U1A	207	172	Exon S
			390	
At2g47890	putative CONSTANS-like B-box zinc finger protein;COLD	325	**497**	
			575	Intron R
At3g01090	putative SNF1-related protein kinase (KIN10);Dark, sugar, hypoxia	342	**117**	
			222	5'SS
At3g01150	polypyrimidine tract-binding protein, putative	195	156	5'SS
			203	
		196	**165**	
			268	3'SS,5'SS
At3g01540	DEAD box RNA helicase (DRH1)	283	135	Exon S
			349	Unknown
			387	
			480	5'SS
At3g04030	myb family transcription factor	139	145	Unknown
			151	3'SS
			164	
			170	3'SS
			462	Unknown
At3g06510	glycosyl hydrolase family 1 protein; COLD	360	256	Unknown
			307	3'SS
			314	
			343	3'SS
			415	Intron R
At3g07740	transcriptional adaptor (ADA2a)	136	**136**	
			240	5'SS
At3g07810	putative / hnRNP	273	124	3'SS
			173	
At3g08505	zinc finger (CCCH-type/C3HC4-type RING finger) family protein	134	197	3'SS
			205	
At3g10300	calcium-binding EF hand family protein;COLD	330	**118**	
			122	Intron R
		331	123	Exon S
			297	Exon S
			316	
At3g10490	no apical eristems (NAM) family protein	138	172	3'SS
			181	
			190	Unknown
At3g11540	Spindly (gibberellin signal transduction protein)	301	110	Exon S
			616	Unknown
			662	
At3g12110C	Actin 11 (ACT11)	63	245	C
			400	C
At3g12250	bZIP family transcription factor	142	**160**	
			203	3'SS
			234	3'SS
		143	**220**	
			329	5'SS

			288	159	3'SS
				189	
At3g12570	expressed protein		289	141	Exon S
				172	Unknown
				230	Unknown
				259	Unknown
				351	
At3g13224	RNA recognition motif (RRM)-containing protein		373	**267**	
				494	Intron R
At3g13570	SC35-like splicing factor		202	190	Exon S
				351	
At3g14230	Transcription factor EREBP like		82	213	5'SS
				216	5'SS
				228	
At3g14740	PHD finger family protein		137	**288**	
				294	3'SS
At3g15030	TCP family transcription factor, putative		140	169	3'SS
				180	
At3g16785	phospholipase D, putative; ABA		355	225	Intron R
				234	
				313	Unknown
At3g16800	protein phosphatase 2C, putative / PP2C, putative;COLD		332	327	Unknown
				367	
				385	3'SS
			333	**313**	
				408	Intron R
At3g17090	Phosphatase-2c		259	**197**	
				214	3'SS
At3g17609	bZIP transcription factor family protein		145	**147**	
				151	3'SS
				187	3'SS
				193	3'SS
At3g19840	WW domain-containing protein		285	**172**	
				207	5'SS
At3g20270	lipid-binding serum glycoprotein		375	195	Exon S
				217	
				247	Unknown
At3g23280	zinc finger (C3HC4-type RING finger) family protein		144	128	Exon S
				201	
				286	Unknown
At3g23830	glycine-rich RNA-binding protein, putative		277	**131**	
				145	3'SS
At3g23900	RNA recognition motif (RRM)-containing protein		272	118	5'SS
				125	
At3g24120	myb family transcription factor		146	**170**	
				179	3'SS
At3g25840	protein kinase family protein		279	**283**	
				341	3'SS, 5'SS
				503	Unknown
At3g26740	light regulated protein CCR-Like Circadian regulated		260	196	3'SS
				207	
At3g26744	ICE1;COLD		411	241	Unknown
				294	3'SS
				345	

Gene ID	Description			
At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia	343	**159**	
			188	Unknown
			308	5'SS
		344	195	3'SS
			200	
At3g44300	nitrilase 2	237	214	
			235	Alt. P
At3g46460	ubiquitin-conjugating enzyme 13 (UBC13)	303	139	5'SS
			179	
At3g47550	zinc finger (C3HC4-type RING finger) family protein	151	228	3'SS
			233	Unknown
			237	
At3g49430	pre-mRNA splicing factor, putative	194	**141**	
			366	3'SS, 5'SS
			544	Unknown
At3g51530	F-box family protein	377	442	Alt. P
			450	
At3g51880	high mobility group protein alpha	141	202	5'SS
			223	
At3g53270	Unknown protein	30	109	Exon S
			212	
			264	3'SS
			270	3'SS,5'SS
			277	3'SS
			360	3'SS
			368	3'SS
			595	Unknown
			674	Intron R
At3g53500	zinc knuckle (CCHC-type) family protein	204	**156**	
			272	Unknown
			376	3'SS
			493	Unknown
At3g53570	protein kinase (AFC1) (AME2)	225	138	3'SS
			179	
At3g54480	SKP1 interacting partner 5 (SKIP5)	135	227	3'SS
			258	
At3g55460	SCL30	220	**135**	
			590	3'SS, 5'SS
			673	Unknown
At3g56860	UBA2a; RNA binding protein; ABA	356	**64**	
			78	Unknown
			330	3'SS
At3g58030	zinc finger (C3HC4-type RING finger) family protein	152	246	3'SS
			252	
At3g59060	basic helix-loop-helix (bHLH) family protein	153	232	3'SS
			238	
At3g61860	arginine/serine-rich splicing factor RSP31	205	**158**	
			556	3'SS, 5'SS
			670	3'SS
At3g62190	DNAJ heat shock N-terminal domain-containing protein	378	**143**	
			218	3'SS
			335	Unknown
At4g01060	myb family transcription factor	154	160	3'SS
			163	
			169	3'SS
At4g01250	WRKY family transcription factor;COLD	366	**213**	
			397	Intron R

At4g02200	drought-induced-19-like 1	384	**115**	
			149	5'SS
At4g02430	SR34b	210	**153**	
			162	3'SS
		212	131	3'SS
			143	
			295	Intron R
At4g02560C	Ld.1 LUMINIDEPENDENS (LD)	66	**239**	
			475	U12 Type
At4g10100	molybdenum cofactor synthesis family protein (Auxin regulated)	261	242	Unknown
			253	
			269	5'SS
			286	Unknown
At4g12790	Unknown protein	36	168	Unknown
			170	Unknown
			183	3'SS
			212	3'SS
			261	Unknown
			341	
At4g13020	serine/threonine-specific protein kinase MHK;COLD	356	**267**	
			350	Intron R
At4g13100	zinc finger (C3HC4-type RING finger) family protein	163	234	3'SS
			242	
At4g13850	Glycine-rich RNA binding protein AtGRP2; Like; COLD; OSMOTIC	381	**252**	
			341	Intron R
At4g14410	basic helix-loop-helix (bHLH) family protein	160	**182**	
			341	3'SS
At4g16420	transcriptional adaptor (ADA2b)	165	183	3'SS
			195	
At4g16845	Vernalization 2 protein (VRN2)	86	**374**	
			396	5'SS
At4g17615	calcineurin B-like protein 1 (CBL1);COLD	329	168	Unknown
			176	
			204	Unknown
			588	Intron R
At4g18020	pseudo-response regulator 2 (APRR2) (TOC2)	167	105	3'SS
			115	
At4g23260	protein kinase family protein;COLD	346	245	Unknown
			285	
			401	Intron R
At4g24740	protein kinase (AFC2)	226	143	Exon S
			309	
		227	152	Exon S
			343	
At4g25500	arginine/serine-rich splicing factor RSP40 (RSP40)	219	**124**	
			382	3'SS, 5'SS
			628	Unknown
At4g25990	expressed protein	166	**174**	
			220	3'SS
At4g26070	mitogen-activated protein kinase kinase (MAPKK) (MKK1) (MEK1);COLD; WOUNDING; BIOTIC	362	**459**	
			548	Intron R
At4g27050	F-box family protein	155	178	Unknown
			187	5'SS
			226	Unknown
			233	Unknown
			352	

At4g27410	no apical meristem (NAM) family protein (RD26);COLD	321	**336**	
			418	Unknown
			446	Intron R
At4g28790	basic helix-loop-helix (bHLH) family protein	161	**176**	
			243	3'SS
At4g29810	MAP kinase kinase 2;COLD	412	155	Unknown
			185	
			212	3'SS
			301	Unknown
At4g31120	SKB1 Methyltransferase	315	**339**	
			432	Intron R
At4g31720	transcription initiation factor IID (TFIID) 23-30kDa subunit (TAF2H) family protein; SALT	369	134	Unknown
			189	3'SS
			206	
			286	Unknown
			303	Unknown
At4g32660	protein kinase (AFC3) (AME3)	228	**199**	
			220	5'SS
At4g32730	myb family transcription factor	158	**184**	
			199	5'SS
At4g33060	peptidyl-prolyl cis-trans isomerase	284	203	Exon S
			290	
			295	Unknown
			300	Unknown
At4g34000	ABA-responsive elements-binding factor (ABF3); ABA	365	186	Unknown
			228	
			320	Intron R
At4g34430	DNA-binding family protein	156	192	3'SS
			197	
At4g34460	GTP binding protein beta subunit; ABA	347	221	3'SS
			233	
		348	160	3'SS
			167	Unknown
			172	Unknown
			175	
			416	Intron R
At4g35450	Ankyrin repeat-containing protein 2	87	**304**	
			350	5'SS
At4g35785	transformer serine/arginine-rich ribonucleoprotein	229	**115**	
			153	Unknown
			281	Unknown
			302	Unknown
			414	3'SS, Intron R
At4g36690	U2 snRNP auxiliary factor large subunit	198	**129**	
			303	3'SS
At4g36730	G-box binding factor 1 (GBF1)	292	151	3'SS
			157	
At4g36960	RNA recognition motif (RRM)-containing protein	374	**228**	
			328	Intron R
			399	Intron R
At4g37180	myb family transcription factor	159	**216**	
			237	3'SS
At4g38510	vacuolar-type H+-ATPase subunit B2	89	216	5'SS
			232	5'SS
			289	
			308	Unknown
At4g38900	bZIP protein	157	**223**	
			249	3'SS

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At4g39260	glycine-rich RNA-binding protein 8 (GRP8) (CCR1)	90	**158**	
			316	5'SS
At5g02470	DP-2 transcription factor, putative (DPA)	187	**165**	
			293	5'SS
At5g02840	myb family transcription factor	188	152	3'SS
			165	Unknown
			174	
At5g04275	MIR172b	379	141	Unknown
			162	
			254	Intron R
At5g04430	Putative RNA binding protein	42	**140**	
			205	3'SS
At5g05550	expressed protein	181	208	Exon S
			307	
			588	Unknown
At5g06160	SF3a60/SAP61 Subunit	222	159	Exon S
			296	
			205	Unknown
At5g06960	bZIP family transcription factor (OBF5)	176	214	3'SS
			230	
At5g08185	npcRNA 78; MIR162a	380	**98**	
			101	3'SS
			167	Exon S
			170	Unknown
			260	Unknown
			263	Unknown
At5g09230	Transcriptional regulator (Sir2)	43	91	Unknown
			112	Exon S
			157	3'SS
			196	
		244	**119**	
			131	5'SS
At5g09790	PHD finger family protein / SET domain-containing protein	301	275	Unknown
			277	5'SS
			281	Unknown
			284	
At5g09880	RNA recognition motif (RRM)-containing protein	298	143	Exon S
			219	3'SS, 5'SS
			270	
			281	Unknown
			299	3'SS, 5'SS
			376	Unknown
			508	Unknown
			634	3'SS
At5g10140	FLC	308	**213**	
			285	3'SS
At5g11200	UAP56a	280	173	Unknown
			220	
			357	3'SS
At5g11330	monooxygenase family protein	299	189	Unknown
			289	Alt. P
			354	Unknown
			360	
At5g12840	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	186	**158**	
			221	5'SS
			471	Unknown
At5g13220	expressed protein	174	**172**	
			224	5'SS

Gene	Description		Position	Type
At5g13730	RNA pol sigma subunit D (sigD)	44	**273**	
			288	3'SS
			201	Unknown
At5g13790	AGL15	312	**461**	
			469	Unknown
			575	Unknown
			584	Unknown
			648	Intron R
At5g15920	heat shock transcription factor 3 (HSTF3)	182	236	3'SS
			241	
At5g18240	myb family transcription factor	168	145	Unknown
			151	3'SS
			163	
			169	3'SS
At5g18620	DNA-dependent ATPase, putative	171	**212**	
			221	5'SS
At5g18800	NADH-ubiquinone oxidoreductase 19 kDa	304	**179**	
			206	3'SS
At5g18830	squamosa promoter-binding protein-like 7 (SPL7)	191	194	5'SS
			271	
At5g19030	RNA recognition motif (RRM)-containing protein	274	**162**	
			182	3'SS
		275	151	3'SS
			164	
At5g20040	IPP transferase 9 (IPT9)	262	146	3'SS
			163	
			206	Unknown
At5g20250	Seed Imbibition protein-like	47	**203**	
			219	Intron R
			313	Intron R
At5g22000	zinc finger (C3HC4-type RING finger) family protein	183	195	5'SS
			222	
At5g23090	TATA-binding protein-associated phosphoprotein Dr1 protein, putative (DR1)	184	**170**	
			211	3'SS
		185	169	3'SS
			212	
At5g23450	diacylglycerol kinase family protein;sphingosine kinase (AtLCBK1); ABA	354	218	3'SS
			254	Unknown
			278	Unknown
			354	
At5g24270	calcineurin B-like protein, putative / calcium sensor homolog (SOS3); SALT	363	299	Unknown
			307	
			337	5'SS
			415	Intron R
			426	Unknown
			540	Unknown
At5g24520	transparent testa glabra 1 protein (TTG1)	169	146	3'SS
			152	
At5g24590	NAC2-like protein/turnip crinkle virus-interacting protein;COLD	320	**521**	
			609	Intron R
At5g25610	dehydration-responsive protein (RD22)	366	**232**	
			607	Intron R
At5g25080	mitogen-activated protein kinase-like;COLD	368	166	5'SS
			167	
		357	312	
			381	Intron R

Gene ID	Description			
At5g28770	bZIP transcription factor family protein	170	174	3'SS
			195	
			258	Unknown
At5g35410	CBL-interacting protein kinase 24 (CIPK24) / serine/threonine protein kinase (SOS2); SALT	370	**115**	
			120	3'SS
At5g35680	initiation factor 1A putative	48	112	Unknown
			191	Unknown
			199	
			228	3'SS
At5g37055	SEF Zn finger HIT type	313	346	Unknown
			355	
			433	Intron R
			524	Intron R
At5g37370	pre-mRNA splicing factor PRP38 family protein (SRL1); SALT	371	182	Unknown
			190	
			285	3'SS
At5g40280	farnesyltransferase beta subunit (ERA1); ABA	340	**187**	
			432	Intron R
At5g41150	Repair endonuclease (RAD1)	49	**292**	
			346	3'SS
At5g43270	squamosa promoter-binding protein-like 2 (SPL2)	189	161	5'SS
			244	
		324	**187**	
			270	5'SS
At5g43910	Unknown Protein	50	190	3'SS
			203	3'SS
			221	
			307	Unknown
At5g45510	Unknown Protein	51	**114**	
			200	3'SS
At5g46110	P/trioseP Translocator precursor	245	124	Unknown
			130	
			138	5'SS
At5g47210	nuclear RNA-binding protein	281	121	Unknown
			133	Exon S
			170	Unknown
			179	
At5g48150	phytochrome A signal transduction 1 (PAT1)	179	192	Unknown
			203	Exon S
			272	Unknown
			283	
At5g48560	putative bHLH transcription factor (aHLH078); COLD	310	**336**	
			400	Intron R
At5g50240	L-isospartyl methyltransferase	254	193	3'SS
			198	3'SS
			201	
At5g52310	Dessication-responsive protein (RD29A) (COR78);COLD	328	**173**	
			398	5'SS
At5g53180	polypyrimidine tract-binding protein	213	140	Exon S
			198	
			201	Unknown
At5g53450	fibrillin fused protein kinase	381	106	3'SS
			189	
At5g54430	universal stress protein (USP) family protein	233	**100**	
			194	3'SS
			198	Unknown
At5g56370	F-box family protein	190	**161**	
			191	3'SS

At5g57110	Ca2+-transporting ATPase-like protein; ABA	352	121	3'SS
			138	
			126	Unknown
At5g57630	CBL-interacting protein kinase 21 (CIPK21);COLD	338	209	5'SS
			253	
			351	Unknown
At5g59300	Ubiquitin-conjugating enzyme 7	300	217	
			226	Alt P
At5g59440	Thymidylate kinase like	246	143	3'SS
			151	
At5g59780	myb family transcription factor (MYB59)	177	**207**	
			239	5'SS
At5g59950	RNA and export factor binding protein, putative;COLD	327	**224**	
			421	Intron R
At5g60580	zinc finger (C3HC4-type RING finger) family protein	172	**141**	
			164	3'SS
At5g63120	ethylene-responsive DEAD box RNA helicase	282	180	Exon S
			366	
			471	Unknown
At5g63870	Ser/Thr Phospahatase	57	131	Unknown
			174	
			185	3'SS
At5g63990	3'(2'),5-bisphosphate nucleotidase;COLD	340	**364**	
			397	Intron R
At5g65050	MADS-box protein AGL31 FLM	58	174	Unknown
			178	Unknown
			205	Unknown
			208	3'SS
			237	3'SS
			241	
			247	Unknown
			324	3'SS
			328	Intron R
			437	5'SS, Intron R
At5g65060	MAF3	309	219	3'SS
			223	3'SS
			228	3'SS
			257	Unknown
			261	
			306	Unknown
			338	Unknown
			342	Unknown
			344	Unknown
			348	Unknown
At5g65430	GF 14 Kappa isoform	264	245	3'SS
			251	
At5g65640	putative bHLH transcription factor (bHLH093);COLD	319	131	3'SS
			135	Unknown
			143	
At5g66010	Putative protein	59	**103**	
			182	3'SS
At5g66210	calcium-dependent protein kinase;COLD	335	119	Exon S
			295	Unknown
			327	
			430	Intron R
At5g67030	zeaxanthin epoxidase (ZEP) (ABA1); ABA; DESSICATION; HEAT	364	177	Intron R
			183	Intron R
			186	

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At5g67580	myb family transcription factor	178	155	5'SS
			197	

Gene ID and description with corresponding primer pairs, predicted product sizes, and types of alternative splicing events detected. For each gene, the RT-PCR product in bold indicates the constitutively spliced transcript. The sequences for each primer pair are shown in Table S6. AS, alternative splicing; 5'SS, alternative 5' splice site; 3'SS, alternative 3' splice site; Exon S, exon skipping; Intron R, intron retention; Alt P, events involving both alternative 5' and 3' splice sites.

Color key:

	Transcription factors
	RNA binding and processing
	Stress-related
	Flowering time
	Stomatal ABA signaling
	Miscellaneous
	Genes removed from the analysis (detection of a single transcript)
	Control genes

Table B. Genes showing significant changes in the ratio of alternative splice variants between wild-type and *sr45-1* seedlings grown under control conditions.

Primer pair	Locus ID	Gene description	AS type	Mean product size (bp)	Col-0, control Mean	SE	*sr45-1*, control Mean	SE	*P*-value
13	At1g79650	DNA repair protein RAD23		155.2	0.206	0.0026	0.286	0.0032	0.000
			3'SS	173.4	0.790	0.0023	0.710	0.0023	0.000
19	At2g32330	Unknown protein		273.2	0.592	0.0056	0.636	0.0086	0.034
21	At2g37340	RS2Z33		121.3	0.829	0.0037	0.902	0.0042	0.001
			3'SS	342.6	0.099	0.0029	0.061	0.0028	0.019
23	At2g39730	Rubisco activase (Auxin regulated)		246.7	0.594	0.0032	0.560	0.0089	0.016
			3'SS	257.5	0.406	0.0032	0.440	0.0089	0.015
30	At3g53270	Unknown protein		212.5	0.058	0.0038	0.011	0.0021	0.000
			3'SS	264.0	0.314	0.0038	0.270	0.0014	0.001
			3'SS, 5'SS	270.2	0.096	0.0067	0.044	0.0033	0.000
			3'SS	277.3	0.093	0.0023	0.055	0.0032	0.000
			3'SS	361.2	0.081	0.0027	0.113	0.0031	0.001
			3'SS	368.0	0.104	0.0029	0.161	0.0069	0.001
			Intron R	678.2	0.122	0.0022	0.232	0.0069	0.000
36	At4g12790	Unknown protein	3'SS	211.5	0.441	0.0017	0.155	0.0019	0.000
				340.5	0.416	0.0071	0.744	0.0002	0.000
42	At5g04430	Putative RNA binding protein		140.3	0.647	0.0080	0.702	0.0094	0.001
			3'SS	204.8	0.353	0.0080	0.298	0.0094	0.001
43	At5g09230	Transcriptional regulator (Sir2)	Exon S	112.3	0.362	0.0205	0.279	0.0191	0.026
59	At5g66010	Putative protein		103.1	0.429	0.0176	0.362	0.0121	0.013
			3'SS	181.7	0.571	0.0176	0.638	0.0121	0.008
72	At2g04790	Expressed protein		166.5	0.790	0.0043	0.727	0.0160	0.048
75	At2g36000	Expressed protein	5'SS	151.1	0.477	0.0017	0.365	0.0035	0.000
				254.7	0.523	0.0017	0.635	0.0035	0.000
89	At4g38510	vacuolar-type H+-ATPase subunit B2	5'SS	215.6	0.368	0.0020	0.267	0.0005	0.000
			5'SS	231.6	0.226	0.0038	0.311	0.0006	0.000
102	At1g27370	squamosa promoter-binding protein-like 10 (SPL10)		163.6	0.652	0.0324	0.838	0.0508	0.010
			3'SS	192.0	0.348	0.0324	0.162	0.0508	0.033
106	At1g49950	DNA-binding protein, putative		192.3	0.884	0.0053	0.815	0.0035	0.000
			5'SS	237.3	0.116	0.0053	0.185	0.0035	0.000
109	At1g77080	MADS affecting flowering 1 (MAF1)	3'SS	117.9	0.177	0.0264	0.119	0.0116	0.034
				157.8	0.823	0.0264	0.881	0.0116	0.032
112	At1g09530	phytochrome interacting factor 3 (PIF3)		230.1	0.845	0.0053	0.777	0.0051	0.000
			5'SS	290.5	0.155	0.0053	0.223	0.0051	0.000
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein		197.1	0.431	0.0073	0.471	0.0173	0.031
			3'SS	221.7	0.265	0.0038	0.204	0.0084	0.000
120	At2g41710	ovule development protein, putative	3'SS	229.8	0.453	0.0086	0.600	0.0029	0.000
				245.5	0.547	0.0086	0.400	0.0029	0.000
126	At2g34830	WRKY family transcription factor	3'SS	199.2	0.388	0.0402	0.139	0.0351	0.002
				216.7	0.597	0.0383	0.849	0.0340	0.001
129	At2g40830	zinc finger (C3HC4-type RING finger) family protein		218.9	0.891	0.0037	0.845	0.0036	0.000
			5'SS	329.3	0.056	0.0028	0.088	0.0032	0.000
132	At2g31370	bZIP transcription factor (POSF21)		193.3	0.713	0.0206	0.804	0.0290	0.026
			Unknown	213.5	0.074	0.0111	0.019	0.0046	0.001
			Unknown	236.2	0.057	0.0063	0.022	0.0134	0.040
136	At3g07740	transcriptional adaptor (ADA2a)		136.1	0.930	0.0256	0.978	0.0069	0.036
			5'SS	240.0	0.070	0.0256	0.022	0.0069	0.046
139	At3g04030	myb family transcription factor	3'SS	151.3	0.064	0.0107	0.028	0.0062	0.008
143	At3g12250	bZIP family transcription factor		219.9	0.926	0.0060	0.881	0.0021	0.001
			5'SS	330.0	0.074	0.0060	0.119	0.0021	0.002

#	Gene	Description	Site						
145	At3g17609	bZIP transcription factor family protein		147.9	0.417	0.0037	0.291	0.0058	0.000
			3'SS	193.8	0.512	0.0063	0.652	0.0038	0.000
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	5'SS	189.5	0.269	0.0084	0.212	0.0024	0.000
				212.6	0.731	0.0084	0.788	0.0024	0.000
149	At2g27230	transcription factor-related	5'SS	206.8	0.242	0.0023	0.275	0.0147	0.015
				244.4	0.758	0.0023	0.725	0.0147	0.018
151	At3g47550	zinc finger (C3HC4-type RING finger) family protein	3'SS	228.0	0.101	0.0092	0.048	0.0059	0.001
				237.8	0.886	0.0121	0.945	0.0050	0.001
154	At4g01060	myb family transcription factor		162.4	0.573	0.0064	0.640	0.0082	0.000
			3'SS	168.3	0.410	0.0141	0.351	0.0093	0.007
155	At4g27050	F-box family protein	Unknown	177.6	0.230	0.0050	0.064	0.0066	0.000
			Unknown	233.3	0.085	0.0088	0.028	0.0024	0.000
				351.9	0.659	0.0026	0.891	0.0081	0.000
161	At4g28790	basic helix-loop-helix (bHLH) family protein		175.9	0.863	0.0375	0.960	0.0046	0.025
			3'SS	242.8	0.137	0.0375	0.040	0.0046	0.017
165	At4g16420	transcriptional adaptor (ADA2b)	3'SS	183.0	0.359	0.0058	0.412	0.0028	0.000
				195.2	0.641	0.0058	0.588	0.0028	0.000
168	At5g18240	myb family transcription factor	3'SS	152.0	0.214	0.0156	0.143	0.0144	0.019
171	At5g18620	DNA-dependent ATPase, putative		212.0	0.745	0.0082	0.709	0.0061	0.004
			5'SS	221.0	0.255	0.0082	0.291	0.0061	0.004
177	At5g59780	myb family transcription factor (MYB59)		208.8	0.922	0.0035	0.964	0.0012	0.002
			5'SS	237.7	0.078	0.0035	0.036	0.0012	0.001
178	At5g67580	myb family transcription factor	5'SS	155.1	0.064	0.0035	0.105	0.0070	0.001
				196.9	0.936	0.0035	0.895	0.0070	0.001
181	At5g05550	expressed protein		306.5	0.247	0.0062	0.116	0.0114	0.000
			Unknown	588.3	0.119	0.0078	0.190	0.0101	0.012
187	At5g02470	DP-2 transcription factor, putative (DPA)		165.4	0.565	0.0444	0.689	0.0354	0.028
			5'SS	293.1	0.435	0.0444	0.311	0.0354	0.020
189	At5g43270	squamosa promoter-binding protein-like 2 (SPL2)	5'SS	161.1	0.339	0.0197	0.432	0.0165	0.005
				245.1	0.661	0.0197	0.568	0.0165	0.012
190	At5g56370	F-box family protein		160.4	0.497	0.0136	0.589	0.0316	0.024
			3'SS	191.3	0.503	0.0136	0.411	0.0316	0.042
193	At1g07350	transformer serine/arginine-rich ribonucleoprotein		200.0	0.526	0.0149	0.351	0.0192	0.004
			3'SS	294.6	0.323	0.0095	0.407	0.0114	0.021
			3'SS	443.7	0.046	0.0019	0.127	0.0040	0.000
195	At3g01150	polypyrimidine tract-binding protein, putative	5'SS	155.4	0.090	0.0063	0.038	0.0021	0.000
				202.7	0.910	0.0063	0.962	0.0021	0.000
198	At4g36690	U2 snRNP auxiliary factor large subunit		128.8	0.564	0.0118	0.479	0.0117	0.021
204	At3g53500	RS2Z32		155.6	0.856	0.0058	0.790	0.0172	0.023
			3'SS	375.8	0.071	0.0025	0.109	0.0074	0.023
205	At3g61860	RS31		158.3	0.681	0.0063	0.875	0.0124	0.000
			3'SS	671.6	0.296	0.0053	0.086	0.0089	0.000
206	At2g21660	glycine-rich RNA-binding protein (GRP7)	Unknown	171.3	0.016	0.0032	0.149	0.0508	0.001
				180.8	0.910	0.0052	0.721	0.0690	0.007
			5'SS	349.3	0.011	0.0006	0.083	0.0125	0.000
209	At2g16940	RNA recognition motif (RRM)-containing protein		136.1	0.587	0.0027	0.769	0.0135	0.000
			3'SS	418.4	0.316	0.0012	0.109	0.0054	0.000
213	At5g53180	polypyrimidine tract-binding protein	Exon S	139.4	0.792	0.0006	0.860	0.0031	0.000
				198.3	0.119	0.0026	0.078	0.0017	0.001
217	At1g16610	SR45	3'SS	154.9	0.229	0.0101	0.315	0.0113	0.000
				175.5	0.758	0.0068	0.666	0.0156	0.002
219	At4g25500	RS40	3'SS, 5'SS	124.2	0.809	0.0026	0.623	0.0194	0.000
				382.1	0.190	0.0026	0.372	0.0175	0.000
224	At1g15200	atPinin		130.0	0.678	0.0049	0.900	0.0066	0.000
			Unknown	235.5	0.067	0.0020	0.014	0.0006	0.000
			3'SS	312.4	0.248	0.0023	0.082	0.0053	0.000
225	At3g53570	protein kinase (AFC1) (AME2)	3'SS	138.6	0.167	0.0019	0.100	0.0033	0.000
				179.2	0.833	0.0019	0.900	0.0033	0.000

#	Gene	Description	Type						
226	At4g24740	protein kinase (AFC2)	Exon S	143.0	0.134	0.0036	0.090	0.0021	0.001
				309.2	0.866	0.0036	0.910	0.0021	0.000
227	At4g24740	protein kinase (AFC2)	Exon S	153.1	0.132	0.0021	0.173	0.0044	0.006
				343.6	0.868	0.0021	0.827	0.0044	0.007
229	At4g35785	transformer serine/arginine-rich ribonucleoprotein		115.0	0.583	0.0053	0.723	0.0286	0.005
			Unknown	281.1	0.054	0.0014	0.014	0.0013	0.000
			Unknown	413.9	0.309	0.0043	0.222	0.0314	0.045
234	At1g55870	CAF1 family ribonuclease	3'SS	151.3	0.148	0.0046	0.114	0.0049	0.007
				170.6	0.852	0.0046	0.886	0.0049	0.002
236	At1g04950	TATA box-binding protein-associated factor		266.9	0.035	0.0030	0.068	0.0032	0.001
243	At2g33830	Auxin regulated protein (Dormancy)	3'SS	139.2	0.014	0.0016	0.538	0.0952	0.000
				147.5	0.986	0.0016	0.462	0.0952	0.001
245	At5g46110	P/trioseP Translocator precursor	Unknown	123.5	0.286	0.0628	0.086	0.0213	0.016
				129.2	0.652	0.0631	0.876	0.0234	0.009
260	At3g26740	light regulated protein CCR-Like Circadian regulated		197.4	0.011	0.0009	0.137	0.0233	0.000
			3'SS	207.0	0.989	0.0009	0.863	0.0233	0.001
261	At4g10100	molybdenum cofactor synthesis family protein (Auxin regulated)		253.3	0.520	0.0274	0.443	0.0130	0.010
			Unknown	286.3	0.171	0.0084	0.235	0.0132	0.001
272	At3g23900	RNA recognition motif (RRM)-containing protein	5'SS	117.3	0.277	0.0046	0.326	0.0076	0.010
				124.3	0.723	0.0046	0.674	0.0076	0.010
275	At5g19030	RNA recognition motif (RRM)-containing protein	3'SS	150.4	0.239	0.0092	0.189	0.0022	0.000
				164.8	0.761	0.0092	0.811	0.0022	0.000
278	At2g02570	atSPF30		152.5	0.940	0.0036	0.973	0.0031	0.001
			3'SS	310.0	0.056	0.0047	0.022	0.0032	0.001
279	At3g25840	protein kinase family protein	3'SS, 5'SS	340.6	0.071	0.0019	0.034	0.0019	0.000
			Unknown	502.9	0.052	0.0041	0.110	0.0050	0.000
282	At5g63120	ethylene-responsive DEAD box RNA helicase	Exon S	179.4	0.784	0.0091	0.818	0.0062	0.045
283	At3g01540	DEAD box RNA helicase (DRH1)	Exon S	134.4	0.879	0.0125	0.936	0.0113	0.002
289	At3g12570	expressed protein	Unknown	258.8	0.107	0.0014	0.073	0.0018	0.000
				350.8	0.694	0.0048	0.757	0.0030	0.000
292	At4g36730	G-box binding factor 1 (GBF1)	3'SS	150.5	0.229	0.0135	0.188	0.0011	0.004
				156.6	0.771	0.0135	0.812	0.0011	0.004
295	At2g02390	glutathione S-transferase zeta1	3'SS	181.4	0.840	0.0026	0.736	0.0012	0.000
				202.3	0.134	0.0007	0.237	0.0013	0.000
298	At5g09880	RNA recognition motif (RRM)-containing protein	Exon S	142.2	0.472	0.0267	0.308	0.0129	0.002
			3'SS, 5'SS	219.0	0.111	0.0056	0.074	0.0040	0.002
			3'SS	634.3	0.005	0.0006	0.099	0.0064	0.000
303	At3g46460	ubiquitin-conjugating enzyme 13 (UBC13)		138.2	0.389	0.0304	0.276	0.0180	0.010
			5'SS	178.9	0.611	0.0304	0.724	0.0180	0.010
309	At5g65060	MAF3		261.6	0.318	0.0034	0.263	0.0077	0.009
314	At2g43410	FPA		406.3	0.406	0.0060	0.259	0.0027	0.000
			Intron R	540.7	0.594	0.0060	0.741	0.0027	0.000
322	At2g33480	putative NAM (no apical meristem)-like protein;COLD	3'SS	321.0	0.139	0.0045	0.056	0.0039	0.000
			Unknown	487.8	0.175	0.0060	0.253	0.0125	0.000
323	At1g76580	Squamosa promoter binding protein-like 16 (SPL16);COLD	3'SS	156.0	0.139	0.0187	0.078	0.0030	0.013
				199.3	0.861	0.0187	0.922	0.0030	0.010
324	At5g43270	squamosa promoter binding protein-like 2;COLD		186.3	0.892	0.0089	0.835	0.0186	0.016
			5'SS	270.1	0.108	0.0089	0.165	0.0186	0.018
332	At3g16800	protein phosphatase 2C, putative / PP2C, putative;COLD	Unknown	326.9	0.068	0.0105	0.144	0.0148	0.007
				367.5	0.869	0.0096	0.713	0.0295	0.000
			3'SS	384.9	0.063	0.0034	0.144	0.0148	0.000
335	At5g66210	calcium-dependent protein kinase;COLD	Unknown	295.2	0.062	0.0007	0.025	0.0050	0.000
			Intron R	431.4	0.892	0.0059	0.954	0.0018	0.000
338	At5g57630	CBL-interacting protein kinase 21 (CIPK21);COLD	Unknown	352.2	0.036	0.0016	0.071	0.0007	0.000
343	At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia		159.2	0.310	0.0036	0.215	0.0069	0.000
			Unknown	188.2	0.097	0.0059	0.036	0.0012	0.000
			5'SS	307.9	0.593	0.0080	0.749	0.0080	0.000

#	Gene	Description	AS type	Size	Col-0	SE	sr45-1	SE	P
345	At1g49730	protein kinase family protein;COLD		236.5	0.764	0.0026	0.667	0.0080	0.000
			Intron R	342.1	0.044	0.0019	0.148	0.0028	0.000
354	At5g23450	diacylglycerol kinase family protein;sphingosine kinase (AtLCBK1); ABA	3'SS	218.4	0.236	0.0027	0.180	0.0039	0.000
				354.2	0.697	0.0030	0.788	0.0038	0.000
356	At3g56860	UBA2a; RNA binding protein; ABA	3'SS	329.9	0.146	0.0096	0.059	0.0091	0.030
360	At3g06510	glycosyl hydrolase family 1 protein; COLD		314.0	0.887	0.0040	0.744	0.0035	0.000
			Intron R	415.2	0.087	0.0038	0.238	0.0026	0.000
364	At5g67030	zeaxanthin epoxidase (ZEP) (ABA1); ABA; DESSICATION; HEAT	Intron R	176.5	0.051	0.0043	0.008	0.0006	0.010
				186.1	0.930	0.0032	0.979	0.0069	0.029
365	At4g34000	ABA-responsive elements-binding factor (ABF3); ABA	Unknown	187.2	0.009	0.0006	0.066	0.0059	0.000
				227.8	0.976	0.0008	0.916	0.0038	0.000
369	At4g31720	transcription initiation factor IID 23-30kDa subunit (TAF2H) family protein; SALT	3'SS	190.0	0.359	0.0035	0.327	0.0008	0.000
372	At1g76460	RNA recognition motif (RRM)-containing protein		258.9	0.870	0.0064	0.817	0.0064	0.000
			3'SS	313.8	0.081	0.0057	0.127	0.0026	0.000
373	At3g13224	RNA recognition motif (RRM)-containing protein		266.8	0.834	0.0065	0.766	0.0055	0.009
			Intron R	494.2	0.166	0.0065	0.234	0.0055	0.022
374	At4g36960	RNA recognition motif (RRM)-containing protein		227.7	0.894	0.0022	0.835	0.0074	0.003
			Intron R	327.3	0.069	0.0020	0.101	0.0033	0.001
376	At1g64625	bHLH transcription factor	Exon S	110.3	0.530	0.0278	0.437	0.0110	0.006
			Unknown	134.7	0.109	0.0029	0.193	0.0066	0.000
			Unknown	154.7	0.100	0.0075	0.059	0.0056	0.002
378	At3g62190	DNAJ heat shock N-terminal domain-containing protein		143.2	0.722	0.0140	0.544	0.0190	0.003
			Unknown	334.6	0.238	0.0147	0.391	0.0224	0.010
380	At5g08185	npcRNA 78; MIR162a	Exon S	167.2	0.187	0.0127	0.131	0.0055	0.021
			Unknown	170.1	0.141	0.0037	0.089	0.0057	0.028
381	At5g53450	Fibrillin fused protein kinase	3'SS	105.7	0.311	0.0111	0.236	0.0035	0.000
				188.2	0.689	0.0111	0.764	0.0035	0.000

The relative abundance of the alternatively-spliced transcripts is presented for each of the products for the wild type (Col-0) and the mutant (*sr45-1*). Significant changes between the two genotypes is measured at $P < 0.05$. Standard errors are the result of three biological repeats. AS, alternative splicing; SE, standard error; 5'SS, alternative 5' splice site; 3'SS, alternative 3' splice site; Exon S, exon skipping; Intron R, intron retention.

Table C. Genes showing significant changes in the ratio of alternative splice variants between wild-type seedlings grown under control and glucose conditions.

Primer pair	Locus ID	Gene description	AS type	Mean product size (bp)	Col-0, control Mean	Col-0, control SE	Col-0, glucose Mean	Col-0, glucose SE	*P-value*
1	At1g02840	SR34		63.4	0.668	0.0053	0.784	0.0462	0.045
			Unknown	502.9	0.268	0.0037	0.174	0.0384	0.040
7	At1g55310	SCL33		199.1	0.827	0.0099	0.876	0.0156	0.038
19	At2g32330	Unknown protein	3'SS	202.9	0.408	0.0056	0.315	0.0125	0.001
				273.2	0.592	0.0056	0.685	0.0125	0.001
21	At2g37340	RS2Z33		121.3	0.829	0.0037	0.929	0.0144	0.000
			3'SS	342.6	0.099	0.0029	0.050	0.0100	0.004
			3'SS	481.0	0.051	0.0015	0.014	0.0039	0.000
23	At2g39730	Rubisco activase (Auxin regulated)		246.7	0.594	0.0032	0.561	0.0091	0.018
			3'SS	257.5	0.406	0.0032	0.439	0.0091	0.017
30	At3g53270	Unknown protein	3'SS	264.0	0.314	0.0038	0.245	0.0087	0.000
			3'SS	361.2	0.081	0.0027	0.120	0.0048	0.000
			3'SS	368.0	0.104	0.0029	0.191	0.0112	0.000
47	At5g20250	Seed Imbittion protein-like		203.3	0.430	0.0049	0.251	0.0056	0.000
			Intron R	218.9	0.551	0.0051	0.738	0.0054	0.000
48	At5g35680	initiation factor 1A putative		199.1	0.871	0.0206	0.821	0.0023	0.041
			3'SS	227.9	0.107	0.0134	0.151	0.0027	0.010
50	At5g43910	Unknown Protein	3'SS	189.6	0.302	0.0067	0.378	0.0053	0.000
				220.4	0.653	0.0065	0.578	0.0029	0.000
51	At5g45510	Unknown Protein		114.1	0.800	0.0052	0.710	0.0100	0.034
			3'SS	200.2	0.200	0.0052	0.290	0.0100	0.026
58	At5g65050	MADS-box protein AGL31 FLM		240.7	0.377	0.0079	0.497	0.0282	0.001
			Intron R	327.9	0.419	0.0058	0.314	0.0335	0.005
59	At5g66010	Putative protein		103.1	0.429	0.0176	0.280	0.0155	0.000
			3'SS	181.7	0.571	0.0176	0.720	0.0155	0.000
70	At1g54360	TATA binding protein associated 6b (TAF6)	5'SS	129.0	0.098	0.0010	0.139	0.0006	0.000
				151.8	0.902	0.0010	0.861	0.0006	0.000
75	At2g36000	Expressed protein	5'SS	151.1	0.477	0.0017	0.416	0.0028	0.000
				254.7	0.523	0.0017	0.584	0.0028	0.000
89	At4g38510	vacuolar-type H+-ATPase subunit B2	5'SS	215.6	0.368	0.0020	0.441	0.0032	0.000
				288.4	0.392	0.0045	0.333	0.0017	0.000
102	At1g27370	squamosa promoter-binding protein-like 10 (SPL10)		163.6	0.652	0.0324	0.832	0.0552	0.012
			3'SS	192.0	0.348	0.0324	0.168	0.0552	0.038
112	At1g09530	phytochrome interacting factor 3 (PIF3)		230.1	0.845	0.0053	0.915	0.0004	0.000
			5'SS	290.5	0.155	0.0053	0.085	0.0004	0.000
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein		197.1	0.431	0.0073	0.394	0.0021	0.032
			3'SS	199.9	0.137	0.0046	0.105	0.0134	0.007
			3'SS	221.7	0.265	0.0038	0.210	0.0006	0.001
			Unknown	289.0	0.122	0.0030	0.222	0.0001	0.000
120	At2g41710	ovule development protein, putative	3'SS	229.8	0.453	0.0086	0.613	0.0139	0.000
				245.5	0.547	0.0086	0.387	0.0139	0.000
126	At2g34830	WRKY family transcription factor	3'SS	199.2	0.388	0.0402	0.141	0.0153	0.002
				216.7	0.597	0.0383	0.831	0.0254	0.001
128	At2g15530	zinc finger (C3HC4-type RING finger) family protein	5'SS	222.2	0.303	0.0004	0.351	0.0110	0.001
129	At2g40830	zinc finger (C3HC4-type RING finger) family protein		218.9	0.891	0.0037	0.846	0.0028	0.000
132	At2g31370	bZIP transcription factor (POSF21)		193.3	0.713	0.0206	0.837	0.0190	0.006
			Unknown	213.5	0.074	0.0111	0.034	0.0072	0.007
136	At3g07740	transcriptional adaptor (ADA2a)		136.1	0.930	0.0256	0.986	0.0009	0.018
			5'SS	240.0	0.070	0.0256	0.014	0.0009	0.017
139	At3g04030	myb family transcription factor		166.4	0.702	0.0257	0.549	0.0319	0.007
			3'SS	169.7	0.076	0.0056	0.157	0.0120	0.001
			Unknown	462.0	0.014	0.0011	0.061	0.0148	0.012

#	Gene	Description	Type						
144	At3g23280	zinc finger (C3HC4-type RING finger) family protein	Exon S	128.0	0.336	0.0038	0.383	0.0225	0.021
				201.2	0.662	0.0039	0.614	0.0232	0.027
145	At3g17609	bZIP transcription factor family protein		147.9	0.417	0.0037	0.386	0.0085	0.007
			3'SS	193.8	0.512	0.0063	0.555	0.0088	0.001
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	5'SS	189.5	0.269	0.0084	0.189	0.0067	0.000
				212.6	0.731	0.0084	0.811	0.0067	0.000
149	At2g27230	transcription factor-related	5'SS	206.8	0.242	0.0023	0.281	0.0025	0.006
				244.4	0.758	0.0023	0.719	0.0025	0.008
154	At4g01060	myb family transcription factor		162.4	0.573	0.0064	0.625	0.0082	0.001
			3'SS	168.3	0.410	0.0141	0.364	0.0136	0.024
155	At4g27050	F-box family protein	Unknown	177.6	0.230	0.0050	0.170	0.0067	0.001
				351.9	0.659	0.0026	0.724	0.0080	0.000
156	At4g34430	DNA-binding family protein	3'SS	191.3	0.490	0.0090	0.282	0.0127	0.001
				197.3	0.510	0.0090	0.718	0.0127	0.001
168	At5g18240	myb family transcription factor	3'SS	170.0	0.145	0.0088	0.219	0.0094	0.023
170	At5g28770	bZIP transcription factor family protein (bZIP63)	3'SS	173.4	0.175	0.0016	0.091	0.0179	0.002
			Unknown	258.2	0.001	0.0000	0.107	0.0018	0.000
171	At5g18620	DNA-dependent ATPase, putative		212.0	0.745	0.0082	0.675	0.0049	0.000
			5'SS	221.0	0.255	0.0082	0.325	0.0049	0.000
172	At5g60580	zinc finger (C3HC4-type RING finger) family protein		141.2	0.860	0.0038	0.810	0.0004	0.001
			3'SS	163.6	0.140	0.0038	0.190	0.0004	0.000
181	At5g05550	expressed protein	Exon S	208.3	0.634	0.0141	0.753	0.0242	0.014
				306.5	0.247	0.0062	0.149	0.0142	0.001
187	At5g02470	DP-2 transcription factor, putative (DPA)		165.4	0.565	0.0444	0.351	0.0014	0.001
			5'SS	293.1	0.435	0.0444	0.649	0.0014	0.003
188	At5g02840	myb family transcription factor	Unknown	163.8	0.131	0.0162	0.044	0.0209	0.026
				173.9	0.695	0.0152	0.807	0.0171	0.002
189	At5g43270	squamosa promoter-binding protein-like 2 (SPL2)	5'SS	161.1	0.339	0.0197	0.446	0.0127	0.003
				245.1	0.661	0.0197	0.554	0.0127	0.006
194	At3g49430	SR34a		140.6	0.832	0.0010	0.913	0.0112	0.002
			3'SS, 5'SS	366.4	0.080	0.0035	0.043	0.0056	0.002
			Unknown	544.2	0.089	0.0025	0.044	0.0056	0.003
198	At4g36690	U2 snRNP auxiliary factor large subunit		128.8	0.564	0.0118	0.645	0.0421	0.038
			3'SS	303.0	0.436	0.0118	0.355	0.0421	0.043
205	At3g61860	RS31		158.3	0.681	0.0063	0.819	0.0242	0.000
			3'SS	671.6	0.296	0.0053	0.159	0.0235	0.001
209	At2g16940	RNA recognition motif (RRM)-containing protein		136.1	0.587	0.0027	0.723	0.0372	0.001
			3'SS	418.4	0.316	0.0012	0.206	0.0287	0.003
212	At4g02430	SR34b	3'SS	131.0	0.347	0.0102	0.291	0.0125	0.014
213	At5g53180	polypyrimidine tract-binding protein	Exon S	139.4	0.792	0.0006	0.869	0.0096	0.000
				198.3	0.119	0.0026	0.073	0.0057	0.000
			Unknown	200.9	0.089	0.0032	0.058	0.0039	0.001
214	At1g31600	2OG-Fe(II) oxygenase family protein		170.0	0.989	0.0027	0.931	0.0261	0.017
			Unknown	645.6	0.007	0.0029	0.048	0.0143	0.007
217	At1g16610	SR45	3'SS	154.9	0.229	0.0101	0.264	0.0085	0.027
222	At5g06160	SF3a60/SAP61 Subunit	Exon S	159.3	0.886	0.0069	0.932	0.0081	0.001
234	At1g55870	CAF1 family ribonuclease	3'SS	151.3	0.148	0.0046	0.088	0.0043	0.000
				170.6	0.852	0.0046	0.912	0.0043	0.000
239	At1G31500	endonuclease/exonuclease/phosphatase family protein		118.5	0.614	0.0038	0.558	0.0028	0.000
			3'SS	134.0	0.386	0.0038	0.442	0.0028	0.000
242	At1g60850	RNA pol subunit		107.4	0.745	0.0090	0.709	0.0036	0.001
			3'SS	118.6	0.255	0.0090	0.291	0.0036	0.001
254	At5g50240	L-isospartyl methyltransferase	3'SS	192.6	0.673	0.0030	0.619	0.0051	0.022
				201.3	0.322	0.0029	0.376	0.0041	0.021
259	At3g17090	Phosphatase-2c		196.5	0.918	0.0021	0.956	0.0010	0.000
			3'SS	213.6	0.082	0.0021	0.044	0.0010	0.000
261	At4g10100	molybdenum cofactor synthesis family protein (Auxin regulated)		253.3	0.520	0.0274	0.462	0.0104	0.037
			5'SS	269.2	0.286	0.0193	0.335	0.0100	0.028
270	At1g53650	RNA-binding protein	5'SS	176.0	0.312	0.0075	0.355	0.0065	0.002
				194.0	0.688	0.0075	0.645	0.0065	0.002
275	At5g19030	RNA recognition motif (RRM)-containing protein	3'SS	150.4	0.239	0.0092	0.178	0.0015	0.000
				164.8	0.761	0.0092	0.822	0.0015	0.000

282	At5g63120	ethylene-responsive DEAD box RNA helicase	Exon S	179.4	0.784	0.0091	0.851	0.0074	0.002
			Unknown	471.2	0.190	0.0083	0.131	0.0064	0.004
283	At3g01540	DEAD box RNA helicase (DRH1)	Exon S	134.4	0.879	0.0125	0.915	0.0020	0.019
285	At3g19840	WW domain-containing protein		171.7	0.428	0.0084	0.582	0.0081	0.031
			5'SS	207.3	0.572	0.0084	0.418	0.0081	0.014
288	At3g12570	expressed protein		159.7	0.376	0.0042	0.416	0.0073	0.005
			3'SS	189.2	0.624	0.0042	0.584	0.0073	0.006
291	At2g46270	G-box binding factor 3 (GBF3)	5'SS	153.7	0.384	0.0057	0.536	0.0134	0.000
				222.2	0.616	0.0057	0.464	0.0134	0.000
292	At4g36730	G-box binding factor 1 (GBF1)	3'SS	150.5	0.229	0.0135	0.180	0.0046	0.001
				156.6	0.771	0.0135	0.820	0.0046	0.002
295	At2g02390	glutathione S-transferase zeta1		181.4	0.840	0.0026	0.767	0.0013	0.000
			3'SS	202.3	0.134	0.0007	0.211	0.0018	0.000
298	At5g09880	RNA recognition motif (RRM)-containing protein	Exon S	142.2	0.472	0.0267	0.637	0.0426	0.004
			Unknown	375.6	0.310	0.0258	0.197	0.0395	0.025
303	At3g46460	ubiquitin-conjugating enzyme 13 (UBC13)	5'SS	138.2	0.389	0.0304	0.279	0.0203	0.012
				178.9	0.611	0.0304	0.721	0.0203	0.011
304	At5g18800	NADH-ubiquinone oxidoreductase 19 kDa		178.9	0.886	0.0070	0.933	0.0015	0.000
			3'SS	205.9	0.114	0.0070	0.067	0.0015	0.000
309	At5g65060	MAF3		261.6	0.318	0.0034	0.404	0.0218	0.002
312	At5g13790	AGL15	Unknown	574.5	0.129	0.0014	0.063	0.0152	0.044
316	At2g28550	AP2 transcription factor like protein;COLD	Unknown	205.9	0.117	0.0208	0.056	0.0105	0.012
				262.0	0.855	0.0199	0.908	0.0078	0.016
323	At1g76580	Squamosa promoter binding protein-like 16 (SPL16);COLD	3'SS	156.0	0.139	0.0187	0.068	0.0127	0.004
				199.3	0.861	0.0187	0.932	0.0127	0.004
329	At4g17615	calcineurin B-like protein 1 (CBL1);COLD	Unknown	164.7	0.112	0.0272	0.054	0.0091	0.024
			Unknown	206.0	0.172	0.0394	0.086	0.0102	0.019
343	At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia		159.2	0.310	0.0036	0.209	0.0119	0.000
			5'SS	307.9	0.593	0.0080	0.684	0.0070	0.000
344	At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia	3'SS	196.3	0.094	0.0024	0.062	0.0043	0.000
				201.2	0.906	0.0024	0.938	0.0043	0.000
354	At5g23450	diacylglycerol kinase family protein;sphingosine kinase (AtLCBK1); ABA		354.2	0.697	0.0030	0.743	0.0022	0.000
358	At1g09000	NPK1-related protein kinase, putative (ANP1); OXIDATIVE; COLD	Unknown	189.7	0.254	0.0390	0.113	0.0124	0.013
				316.6	0.591	0.0688	0.838	0.0275	0.017
360	At3g06510	glycosyl hydrolase family 1 protein; COLD		314.0	0.887	0.0040	0.924	0.0088	0.003
369	At4g31720	transcription initiation factor IID (TFIID) 23-30kDa subunit (TAF2H) family protein; SALT	Unknown	134.8	0.020	0.0003	0.056	0.0017	0.000
			3'SS	190.0	0.359	0.0035	0.297	0.0038	0.000
370	At5g35410	CBL-interacting protein kinase 24 (CIPK24) / serine/threonine protein kinase (SOS2); SALT		115.7	0.674	0.0057	0.726	0.0136	0.026
			3'SS	120.6	0.326	0.0057	0.274	0.0136	0.028
372	At1g76460	RNA recognition motif (RRM)-containing protein		258.9	0.870	0.0064	0.788	0.0036	0.000
			3'SS	313.8	0.081	0.0057	0.131	0.0007	0.000
374	At4g36960	RNA recognition motif (RRM)-containing protein	Intron R	398.9	0.037	0.0004	0.081	0.0097	0.001
375	At3g20270	lipid-binding serum glycoprotein	Exon S	196.2	0.464	0.0096	0.606	0.0152	0.000
				216.9	0.303	0.0104	0.206	0.0114	0.000
			Unknown	246.9	0.234	0.0032	0.187	0.0077	0.007
383	At2g43640	signal recognition particle 14 kDa family protein (SRP14)	5'SS	169.2	0.123	0.0074	0.192	0.0072	0.000
				215.1	0.850	0.0032	0.777	0.0069	0.000

The relative abundance of the alternatively-spliced transcripts is presented for each of the products for the wild type (Col-0) grown under control and glucose conditions. Significant changes between control and glucose conditions is measured at $P <$ 0.05. Standard errors are the result of three biological repeats. AS, alternative splicing; SE, standard error; 5'SS, alternative 5' splice site; 3'SS, alternative 3' splice site; Exon S, exon skipping; Intron R, intron retention.

Table D. Genes showing significant changes in the ratio of alternative splice variants between *sr45-1* seedlings grown under control and glucose conditions.

Primer pair	Locus ID	Gene description	AS type	Mean product size (bp)	*sr45-1*, control		*sr45-1*, glucose		*P-value*
					Mean	SE	Mean	SE	
19	At2g32320	Unknown protein	3'SS	202.9	0.364	0.0086	0.313	0.0195	0.023
				273.2	0.636	0.0086	0.687	0.0195	0.020
30	At3g53270	Unknown protein	3'SS	264.0	0.270	0.0014	0.196	0.0060	0.000
			3'SS	368.0	0.161	0.0069	0.208	0.0131	0.008
36	At4g12790	Unknown protein	Unknown	167.4	0.021	0.0004	0.069	0.0193	0.004
			3'SS	182.7	0.068	0.0016	0.117	0.0234	0.012
				340.5	0.744	0.0002	0.609	0.0645	0.026
42	At5g04430	Putative RNA binding protein		140.3	0.702	0.0094	0.662	0.0066	0.004
			3'SS	204.8	0.298	0.0094	0.338	0.0066	0.004
43	At5g09230	Transcriptional regulator (Sir2)	Exon S	112.3	0.279	0.0191	0.358	0.0280	0.033
47	At5g20250	Seed Imbitition protein-like		203.3	0.429	0.0064	0.290	0.0294	0.000
			Intron R	218.9	0.546	0.0080	0.693	0.0318	0.000
48	At5g35680	initiation factor 1A putative		199.1	0.849	0.0138	0.800	0.0156	0.044
			3'SS	227.9	0.126	0.0074	0.174	0.0106	0.010
50	At5g43910	Unknown Protein	3'SS	189.6	0.309	0.0049	0.382	0.0075	0.000
				220.4	0.647	0.0047	0.575	0.0049	0.000
58	At5g65050	MADS-box protein AGL31 FLM		240.7	0.335	0.0105	0.442	0.0041	0.001
			Intron R	327.9	0.458	0.0056	0.374	0.0087	0.020
59	At5g66010	Putative protein		103.1	0.362	0.0121	0.212	0.0074	0.000
			3'SS	181.7	0.638	0.0121	0.788	0.0074	0.000
70	At1g54360	TATA binding protein associated 6b (TAF6)	5'SS	129.0	0.119	0.0029	0.170	0.0032	0.000
				151.8	0.881	0.0029	0.830	0.0032	0.000
72	At2g04790	Expressed protein		166.5	0.727	0.0160	0.907	0.0179	0.000
			5'SS	189.9	0.273	0.0160	0.093	0.0179	0.000
75	At2g36000	Expressed protein	5'SS	151.1	0.365	0.0035	0.399	0.0081	0.001
				254.7	0.635	0.0035	0.601	0.0081	0.001
89	At4g38510	vacuolar-type H+-ATPase subunit B2	5'SS	231.6	0.311	0.0006	0.345	0.0066	0.000
				288.4	0.402	0.0011	0.360	0.0045	0.000
112	At1g09530	phytochrome interacting factor 3 (PIF3)		230.1	0.777	0.0051	0.850	0.0034	0.000
			5'SS	290.5	0.223	0.0051	0.150	0.0034	0.000
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein		197.1	0.471	0.0173	0.380	0.0049	0.000
			Unknown	289.0	0.148	0.0108	0.265	0.0039	0.000
120	At2g41710	ovule development protein, putative	3'SS	229.8	0.600	0.0029	0.710	0.0237	0.001
				245.5	0.400	0.0029	0.290	0.0237	0.001
128	At2g15530	zinc finger (C3HC4-type RING finger) family protein	5'SS	222.2	0.326	0.0067	0.379	0.0051	0.001
			Unknown	380.7	0.163	0.0033	0.126	0.0052	0.000
129	At2g40830	zinc finger (C3HC4-type RING finger) family protein		218.9	0.845	0.0036	0.807	0.0036	0.000
131	At2g38880	histone-like transcription factor (CBF/NF-Y) family protein		311.8	0.830	0.0046	0.800	0.0031	0.000
			5'SS	373.5	0.170	0.0046	0.200	0.0031	0.000
139	At3g04030	myb family transcription factor		166.4	0.764	0.0276	0.628	0.0375	0.017
			3'SS	169.7	0.091	0.0158	0.170	0.0103	0.002
143	At3g12250	bZIP family transcription factor		219.9	0.881	0.0021	0.911	0.0080	0.011
			5'SS	330.0	0.119	0.0021	0.089	0.0080	0.019
144	At3g23280	zinc finger (C3HC4-type RING finger) family protein	Exon S	128.0	0.341	0.0045	0.394	0.0058	0.012
				201.2	0.656	0.0046	0.603	0.0057	0.018
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	5'SS	189.5	0.212	0.0024	0.164	0.0075	0.001
				212.6	0.788	0.0024	0.836	0.0075	0.001
151	At3g47550	zinc finger (C3HC4-type RING finger) family protein	3'SS	228.0	0.048	0.0059	0.106	0.0043	0.000
				237.8	0.945	0.0050	0.883	0.0082	0.001
156	At4g34430	DNA-binding family protein	3'SS	191.3	0.446	0.0471	0.281	0.0225	0.002
				197.3	0.554	0.0471	0.719	0.0225	0.003

#	Gene	Description	Type						
158	At4g32730	myb family transcription factor		184.2	0.631	0.0110	0.683	0.0043	0.000
			5'SS	199.3	0.369	0.0110	0.317	0.0043	0.000
161	At4g28790	basic helix-loop-helix (bHLH) family protein		175.9	0.960	0.0046	0.857	0.0014	0.020
			3'SS	242.8	0.040	0.0046	0.143	0.0014	0.011
165	At4g16420	transcriptional adaptor (ADA2b)	3'SS	183.0	0.412	0.0028	0.375	0.0016	0.000
				195.2	0.588	0.0028	0.625	0.0016	0.000
168	At5g18240	myb family transcription factor	3'SS	170.0	0.140	0.0286	0.228	0.0071	0.009
170	At5g28770	bZIP transcription factor family protein		194.1	0.842	0.0086	0.784	0.0267	0.036
			Unknown	258.2	0.007	0.0005	0.107	0.0334	0.000
171	At5g18620	DNA-dependent ATPase, putative		212.0	0.709	0.0061	0.666	0.0063	0.001
			5'SS	221.0	0.291	0.0061	0.334	0.0063	0.002
172	At5g60580	zinc finger (C3HC4-type RING finger) family protein		141.2	0.852	0.0013	0.802	0.0119	0.001
			3'SS	163.6	0.148	0.0013	0.198	0.0119	0.000
176	At5g06960	bZIP family transcription factor (OBF5)	3'SS	214.3	0.224	0.0088	0.260	0.0059	0.027
				230.2	0.776	0.0088	0.740	0.0059	0.025
177	At5g59780	myb family transcription factor (MYB59)		208.8	0.964	0.0012	0.928	0.0107	0.006
			5'SS	237.7	0.036	0.0012	0.072	0.0107	0.003
178	At5g67580	myb family transcription factor	5'SS	155.1	0.105	0.0070	0.074	0.0073	0.005
				196.9	0.895	0.0070	0.926	0.0073	0.004
187	At5g02470	DP-2 transcription factor, putative (DPA)		165.4	0.689	0.0354	0.496	0.0261	0.003
			5'SS	293.1	0.311	0.0354	0.504	0.0261	0.002
189	At5g43270	squamosa promoter-binding protein-like 2 (SPL2)	5'SS	161.1	0.432	0.0165	0.560	0.0242	0.002
				245.1	0.568	0.0165	0.440	0.0242	0.001
190	At5g56370	F-box family protein		160.4	0.589	0.0316	0.678	0.0342	0.038
			3'SS	191.3	0.411	0.0316	0.322	0.0342	0.033
202	At3g13570	SCL30a	Exon S	189.9	0.897	0.0066	0.857	0.0136	0.016
				351.2	0.103	0.0066	0.143	0.0136	0.019
204	At3g53500	zinc knuckle (CCHC-type) family protein		155.6	0.790	0.0172	0.859	0.0272	0.020
206	At2g21660	glycine-rich RNA-binding protein (GRP7)	Unknown	171.3	0.149	0.0508	0.054	0.0050	0.021
				180.8	0.721	0.0690	0.842	0.0175	0.046
212	At4g02430	SR34b	3'SS	131.0	0.322	0.0027	0.247	0.0170	0.002
			Intron R	295.2	0.058	0.0025	0.114	0.0070	0.000
213	At5g53180	polypyrimidine tract-binding protein	Exon S	139.4	0.860	0.0031	0.894	0.0114	0.013
217	At1g16610	SR45	3'SS	154.9	0.315	0.0113	0.388	0.0102	0.002
				175.5	0.666	0.0156	0.585	0.0175	0.002
219	At4g25500	RS40		124.2	0.623	0.0194	0.680	0.0240	0.042
			3'SS, 5'SS	382.1	0.372	0.0175	0.315	0.0231	0.040
222	At5g06160	SF3a60/SAP61 Subunit	Exon S	159.3	0.897	0.0058	0.928	0.0047	0.009
223	At2g29210	splicing factor PWI domain-containing protein		151.3	0.714	0.0148	0.784	0.0189	0.005
			3'SS	201.9	0.286	0.0148	0.216	0.0189	0.005
234	At1g55870	CAF1 family ribonuclease	3'SS	151.3	0.114	0.0049	0.072	0.0076	0.001
				170.6	0.886	0.0049	0.928	0.0076	0.001
235	At1g69080	universal stress protein (USP) family protein	5'SS	162.5	0.094	0.0091	0.060	0.0014	0.006
				204.5	0.906	0.0091	0.940	0.0014	0.006
243	At2g33830	Auxin regulated protein (Dormancy)	3'SS	139.2	0.538	0.0952	0.147	0.0899	0.004
				147.5	0.462	0.0952	0.853	0.0899	0.003
244	At5g09230	SIR2-family Protein		119.2	0.772	0.0109	0.826	0.0119	0.018
			5'SS	130.4	0.228	0.0109	0.174	0.0119	0.024
260	At3g26740	light regulated protein CCR-Like Circadian regulated	3'SS	197.4	0.137	0.0233	0.058	0.0208	0.007
				207.0	0.863	0.0233	0.942	0.0208	0.007
270	At1g53650	RNA-binding protein	5'SS	176.0	0.298	0.0059	0.340	0.0075	0.003
				194.0	0.702	0.0059	0.660	0.0075	0.003
275	At5g19030	RNA recognition motif (RRM)-containing protein	3'SS	150.4	0.189	0.0022	0.150	0.0011	0.000
				164.8	0.811	0.0022	0.850	0.0011	0.001
279	At3g25840	protein kinase family protein		282.9	0.856	0.0052	0.902	0.0024	0.000
			unknown	502.9	0.110	0.0050	0.069	0.0003	0.000
280	At5g11200	UAP56a		219.3	0.853	0.0035	0.894	0.0193	0.037
			3'SS	356.4	0.145	0.0032	0.104	0.0191	0.033

282	At5g63120	ethylene-responsive DEAD box RNA helicase	Exon S	179.4	0.818	0.0062	0.886	0.0161	0.002
			Unknown	471.2	0.170	0.0048	0.105	0.0139	0.001
283	At3g01540	DEAD box RNA helicase (DRH1)	Exon S	134.4	0.936	0.0113	0.978	0.0048	0.012
285	At3g19840	WW domain-containing protein		171.7	0.431	0.0717	0.616	0.0190	0.013
			5'SS	207.3	0.569	0.0717	0.384	0.0190	0.005
288	At3g12570	expressed protein	3'SS	159.7	0.400	0.0113	0.456	0.0052	0.001
				189.2	0.600	0.0113	0.544	0.0052	0.001
289	At3g12570	expressed protein		350.8	0.757	0.0030	0.721	0.0101	0.003
291	At2g46270	G-box binding factor 3 (GBF3)	5'SS	153.7	0.379	0.0171	0.509	0.0067	0.000
				222.2	0.621	0.0171	0.491	0.0067	0.000
295	At2g02390	glutathione S-transferase zeta1		181.4	0.736	0.0012	0.664	0.0018	0.000
			3'SS	202.3	0.237	0.0013	0.317	0.0013	0.000
298	At5g09880	RNA recognition motif (RRM)-containing protein	Exon S	142.2	0.308	0.0129	0.390	0.0068	0.034
309	At5g65060	MAF3		261.6	0.263	0.0077	0.356	0.0006	0.001
314	At2g43410	FPA		406.3	0.259	0.0027	0.345	0.0077	0.000
			Intron R	540.7	0.741	0.0027	0.655	0.0077	0.000
316	At2g28550	AP2 transcription factor like protein;COLD	Unknown	205.9	0.085	0.0074	0.044	0.0013	0.037
322	At2g33480	putative NAM (no apical meristem)-like protein;COLD		399.6	0.618	0.0088	0.657	0.0033	0.008
			Unknown	487.8	0.253	0.0125	0.195	0.0083	0.003
324	At5g43270	squamosa promoter binding protein-like 2 (emb|CAB56576.1);COLD		186.3	0.835	0.0186	0.891	0.0022	0.018
			5'SS	270.1	0.165	0.0186	0.109	0.0022	0.021
327	At5g59950	RNA and export factor binding protein, putative;COLD		225.0	0.894	0.0007	0.929	0.0071	0.005
			Intron R	421.4	0.106	0.0007	0.071	0.0071	0.004
329	At4g17615	calcineurin B-like protein 1 (CBL1);COLD	Unknown	164.7	0.072	0.0052	0.010	0.0013	0.002
332	At3g16800	protein phosphatase 2C, putative / PP2C, putative;COLD	Unknown	326.9	0.144	0.0148	0.025	0.0011	0.000
				367.5	0.713	0.0295	0.901	0.0028	0.000
			3'SS	384.9	0.144	0.0148	0.074	0.0040	0.001
335	At5g66210	calcium-dependent protein kinase;COLD	Unknown	295.2	0.025	0.0050	0.056	0.0046	0.001
			Intron R	431.4	0.954	0.0018	0.917	0.0021	0.000
346	At4g23260	protein kinase family protein;COLD	Unknown	243.0	0.688	0.0208	0.530	0.0056	0.021
				284.8	0.097	0.0067	0.194	0.0046	0.020
358	At1g09000	NPK1-related protein kinase, putative (ANP1); OXIDATIVE; COLD	unknown	189.7	0.328	0.0563	0.169	0.0104	0.016
				316.6	0.610	0.0640	0.802	0.0322	0.045
360	At3g06510	glycosyl hydrolase family 1 protein; COLD		314.0	0.744	0.0035	0.795	0.0038	0.000
			Intron R	415.2	0.238	0.0026	0.191	0.0044	0.014
365	At4g34000	ABA-responsive elements-binding factor (ABF3); ABA	Unknown	187.2	0.066	0.0059	0.018	0.0014	0.000
				227.8	0.916	0.0038	0.967	0.0013	0.000
372	At1g76460	RNA recognition motif (RRM)-containing protein		258.9	0.817	0.0064	0.773	0.0064	0.001
375	At3g20270	lipid-binding serum glycoprotein	Exon S	196.2	0.457	0.0106	0.540	0.0044	0.001
				216.9	0.303	0.0046	0.240	0.0061	0.002
376	At1g64625	bHLH transcription factor	Exon S	110.3	0.437	0.0110	0.514	0.0066	0.014
			Unknown	134.7	0.193	0.0066	0.163	0.0091	0.029
			Unknown	154.7	0.059	0.0056	0.094	0.0095	0.004
381	At5g53450	fibrillin fused protein kinase	3'SS	105.7	0.236	0.0035	0.275	0.0078	0.006
				188.2	0.764	0.0035	0.725	0.0078	0.010
383	At2g43640	signal recognition particle 14 kDa family protein / SRP14 family protein		215.1	0.876	0.0042	0.838	0.0023	0.001

The relative abundance of the alternatively-spliced transcripts is presented for each of the products for the *sr45-1* mutant grown under control and glucose conditions. Significant changes between control and glucose conditions is measured at $P < 0.05$. Standard errors are the result of three biological repeats. AS, alternative splicing; SE, standard error; 5'SS, alternative 5' splice site; 3'SS, alternative 3' splice site; Exon S, exon skipping; Intron R, intron retention.

Table E. Sequences of the primers used to detect the alternative splicing events included in the RT-PCR panel.

Primer pair	Locus ID	Gene description	5' Forward primer	3' Reverse primer
1	At1g02840	pre-mRNA splicing factor SF2 (SR1)	GGCCCCCGCTCGAAGTCAAGG	GGTAGAGGAGATCTTGATCTTG
2	At1g04400	Cryptochrome 2 apoprotein (CRY2) (PHH1)	GGAAGAAAGAAGAGTCAAG	GGATTATCCTCAATCCTTAGG
3	At1g09140	SF2/ASF-like splicing modulator (SRp30)	CCTGCTAGATCCATTTCCCC	GATCTTGATCTTGATTTTG
7	At1g55310	SC35-like splicing factor (SCL33)	GGAAGTCGTTTGAGCAGTTTGG	GGTTTCTTTCTGTTCTCTTCTG
13	At1g79650	DNA repair protein RAD23	GGAGAACAAAGTTACGGAGG	GGAACAGGTGAAGACTGGG
19	At2g32330	Unknown protein	CTTTTGGAATGAGCACTCC	CCGTAGGCAAAGGCAATATCC
21	At2g37340	Unknown protein (RSZ33)	GGACCCGAGACCTTGAACG	GGTAATGTCTTGCATCATCAG
23	At2g39730	Rubisco activase (Auxin regulated)	CCTCCCGTGTTCGAGCAACCC	CCGTTGGATCAAAGTTTTCAGCC
30	At3g53270	Unknown protein	CCAACATCATCATCTTCTC	CCACAAACTCGTCTATCAGC
36	At4g12790	Unknown protein	GGTTTTGAGAGCAAAGAAAAACG	GGTTAACAACATGCATTGTTCG
42	At5g04430	Putative RNA binding protein	CCTTCGAGGAGCAGATGCGGGC	CCTCCAGAACCGTTGGGTGC
43	At5g09230	Transcriptional regulator (Sir2)	CATCCGCCATTAACGACCTC	CTCACAAACCGTCTGCATCC
44	At5g13730	RNA pol sigma subunit D (sigD)	GGGTACCAAGGCAAAGGTTTG	GCAATCTCTTCACAGCTTGG
47	At5g20250	Seed Imbitition protein-like	CCATTCAAATCTCACATTCCC	CCCGTCGGAAATACGAACCGCC
48	At5g35680	initiation factor 1A putative	CCTAATCACTTTCAACAACTC	CCGTCAATGCACATAACGTC
49	At5g41150	Repair endonuclease (RAD1)	CCATCCTGACATGGGTTTTGTC	CCAGTTCCTTTCTTCCGCCTGC
50	At5g43910	Unknown Protein	GGTTGTGTGATGCTTGAGAG	GGTTGTGTCTATGAGTTCCG
51	At5g45510	Unknown Protein	GGAGAAAGTTGTTGTAGGAG	GGTGGAGGTACATCACTGTGG
57	At5g63870	Ser/Thr Phospahatase	GGTCCAAGATCCAAGCTTTGG	GGGAAGGAGGTAGATTCCATG
58	At5g65050	MADS-box protein AGL31 FLM	CAAAGATCATTGATCGTTAC	CTTCACTTCCCCCATCATTAGTTC
59	At5g66010	Putative protein	GGCGGCAGGTCATGTACGG	GGGAAGACCCCTGAGGCGAACG
63	At3g12110C	Actin 11 (ACT11)	GGCTCCTCTCAACCCCAAGG	GGTAATCAGTAAGGTCACGG
66	At4g02560C	Ld.1 LUMINIDEPENDENS (LD)	CGCAAATTGATTCGCAGAGTC	CCACCATCTGTACTTTCCTGC
68	At1g23970	Unknown Protein (Auxin regulated)	CTTGCACATGATCCCGATCC	CCATCGCCCAATGCGCCACC
70	At1g54360	TATA binding protein associated 6b (TAF6)	CCTTCCATTCCACAGAATTC	CCAGATTGAGTCAAAGCCCAC
72	At2g04790	Expressed protein	CCCTGAAAGCATAGAAGCAGC	CCCATGACTTATTAAACTCC
73	At2g21620	RD2 protein (Auxin regulated)	CCGGAACTTGAGAGAGAGAGC	CCATGAGGGCTTGGCTCGTC
75	At2g36000	Expressed protein	CTCGTTTAGTTTGGAGAATC	CTTCATCAGCATTCATTAC
82	At3g14230	Transcription factor EREBP like	CCTTTCGTCTTCACCGCAAC	CCAAGCCATTCTCGGGAGCC
86	At4g16845	Vernalization 2 protein (VRN2)	GGATTGTTCTTGTCCATTTTG	GCAAGCTCTGTTGCCTCGGG
87	At4g35450	Ankyrin repeat-containing protein 2	CCACCACAACATTGTCTTTTC	CCAGCGTTAGGAATAGATCTC
89	At4g38510	vacuolar-type H+-ATPase subunit B2	CCTCTACACAGATCTTTGGATTC	CTTGACCACGTCGGGTAGTTCC
90	At4g39260	glycine-rich RNA-binding protein 8 (GRP8) (CCR1)	GGTGCTTTGTCGGCGGCCTTG	GGCTTTCTCGTCCTTGAAGG
102	At1g27370	squamosa promoter-binding protein-like 10 (SPL10)	CCTCCTTCTCACTCTCGCTC	CCCACGGGAACGAAGATACC
103	At1g30500	CCAAT-binding transcription factor	CCAACCACCAATCCCTTCTGC	CTTGCTCTTGATTGTCTGCGCC
105	At1g33060	no apical meristem (NAM) family protein	GGCTAATTTAAGTGCTCAGGGG	GCTACATTTCGCATCACTTG
106	At1g49950	DNA-binding protein, putative	CCTTGTATTCGCATTAACCC	CCAGGTCCATGCTTTATAACTCC
107	At1g59750	auxin-responsive factor (ARF1)	CCGTCTTCACAACCTCAGCC	CCCACCATGACCAAACGTAGC
108	At1g30200	F-box family protein	GGAACCGTTTGGGACTGTG	GCAGATTCATTCGACGCAGG
109	At1g77080	MADS affecting flowering 1 (MAF1)	GATCTTGAAGAAAAAATTCAG	CCTAGCTCTACTTACGGACAG
110	At1g72050	zinc finger (C2H2 type) family protein	GGTTGATGAGGAGTCTTCAAG	GGACACTTAAAGAGCTTCCC
111	At1g61660	basic helix-loop-helix (bHLH) family protein	GGACATGGTCTATGTCTCGTCC	GCTTTAGAGTCTCCAAGCTG
112	At1g09530	phytochrome interacting factor 3 (PIF3)	CCGTCTTGTGCGTCGATTTG	CCTGTCTTGAGCAGATTCAAGC

113	At1g72650	myb family transcription factor	GGTTTATTGCATTCTGATAATG	GGTGGACTTGATCCTCCTC
116	At1g18660	zinc finger (C3HC4-type RING finger) family protein	GGATTCACCTTTAGTGACTG	GAGACTCTTTGAATGATTGG
117	At2g22670	indoleacetic acid-induced protein 8 (IAA8)	GAAACTGAAGATCATGAAGGG	GAGACGAAATATCTTGAAGG
118	At2g02960	zinc finger (C3HC4-type RING finger) family protein	GGGGAGCTTTCACCAATTAG	GCCTTATCATTAACCACCGG
120	At2g41710	ovule development protein, putative	GGCTGCTGCTAGAGCTTACG	GGTCCAGGCATACGACTGG
121	At2g18300	basic helix-loop-helix (bHLH) family protein	GGATGCAATAAGGTCACAGG	GGTCTAACGGAAACAATGG
122	At2g36010	E2F transcription factor-3 (E2F3)	CCAAAGATGGAATGCTGGACC	CTCAGGTCTCTTAATCTTTCC
124	At2g37060	CCAAT-box binding transcription factor, putative	CCCTCTCTGCCTCCTCTCTTCC	CCCATTAGCAGGAAGACCTC
125	At2g46790	timing of CAB expression 1-like protein (TL1)	GGATTTGCCATCTATATCTGG	GCTTTGAGCATGAGCAGTAGG
126	At2g34830	WRKY family transcription factor	CCGCTATGAACAGCCGGTCC	CCATGGGTGGTTATGCTCAG
127	At2g43010	phytochrome-interacting factor 4 (PIF4)	CCGGTTATGGATCAGTCTGC	CCGTCGGTGGTCTTCGTCGGC
128	At2g15530	zinc finger (C3HC4-type RING finger) family protein	CCCAGAAATTGAAACTACC	CCCTTGCATTGGATTCATACC
129	At2g40830	zinc finger (C3HC4-type RING finger) family protein	CTAGGGTTTCTCATTCCGATCC	CCGTGAAGGCGGACAGCACGG
130	At2g38185	zinc finger (C3HC4-type RING finger) family protein	GCTTGATTACTCAGAACCAGGG	GAACCGCAGAACCTTGTGG
131	At2g38880	histone-like transcription factor (CBF/NF-Y) family protein	GGCAACATTAGGATTTGAGG	GGAAACAATAAACCAAACG
132	At2g31370	bZIP transcription factor (POSF21)	CCAACAGCAGCAGCAGCAAC	CCAAAAGGTTTCTTCTACGTACC
133	At2g32250	far-red impaired responsive protein, putative	CCCATCGATTTCTTCCATCC	CTTCGATGTCATAGCAGCC
134	At3g08505	zinc finger (CCCH-type/C3HC4-type RING finger) family protein	CTATCAGAGTTCCTTGGTGGCC	CATTGAGTTTCTCCTGTGTCC
135	At3g54480	SKP1 interacting partner 5 (SKIP5)	GGAGTTACATGAGATCTCAAAG	GATAAATCCTTGACAAAACGG
136	At3g07740	transcriptional adaptor (ADA2a)	GGGAAGAAGTGCGAGCTTGG	GGTGGAAATTGATGCCGCAG
137	At3g14740	PHD finger family protein	CCGGAAGTTTACTTTGAGGATCC	CAACAATCTTGTACTTTCC
138	At3g10490	no apical meristem (NAM) family protein	GGAATGGGCTGATGATGAAGG	GGTCCGATTCGATATCCAGTGG
139	At3g04030	myb family transcription factor	GCACTGCAAATGCAAATAGAGG	GGCAGCTTCGATCCCAGCTG
140	At3g15030	TCP family transcription factor, putative	GAAGAGGAGCAGCACGACGG	GGCCATTGACTACACAAACCG
141	At3g51880	high mobility group protein alpha	GGGAGTGATGAATCTGAAAAG	GGATCTGCAGTTAAGCTTGAG
142	At3g12250	bZIP family transcription factor	GGACAACCTTTTACTCAGACAGG	GGTATCAGCCATACTAGTTTCTG
143	At3g12250	bZIP family transcription factor	CCCTCTAGTGTGAAACTCTGC	CCTTTGATCGATCACTGGAATC
144	At3g23280	zinc finger (C3HC4-type RING finger) family protein	GCACAGTGATGCCTTTGTGG	GGCTGTCACCTTCAGTCGAAGG
145	At3g17609	bZIP transcription factor family protein	CCAACGACCCAATGGGAACTC	CGTTTAGCTGTAGAGACTCC
146	At3g24120	myb family transcription factor	CCAAGTCACCGAAGCTCTAC	CCAGCAAAAGTAGCAGCTTGC
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	CCGTTTCTCGCTTCTTTTTCTC	CCTCTACAACACCTTTGGTACC
149	At2g27230	transcription factor-related	GGCATGTCGGAGTATGATCG	GACCTTAAAGCTTCTCTTAG
151	At3g47550	zinc finger (C3HC4-type RING finger) family protein	CCTTCTTCGTCGTCGCTGGTTTTC	CCTTAACGGAGAATCATTGCC
152	At3g58030	zinc finger (C3HC4-type RING finger) family protein	CCATCTCTATTCGTAATTAGC	CCCAAGATTCAAATCCAGGTTC
153	At3g59060	basic helix-loop-helix (bHLH) family protein	CCATCCCGGTTTAGTATGTC	CCTATTTTACCCATATGAAGACTGTC
154	At4g01060	myb family transcription factor	GGTGCCGTTTGACATGGATAACC	GTCACCGACAAGCTTATGCATTC
155	At4g27050	F-box family protein	GGTTCTTTCTTTGTGAACAAC	GGGAAACTCCTTAGACAGCAG
156	At4g34430	DNA-binding family protein	GGGCTTCCATTAAATCTCTTCACAG	GGAGACCTCTTCTGCTTTTTTG
157	At4g38900	bZIP protein	GGGAACTCGGGTGCTGCTTTTAG	GGTAGCCTCGGTCTGAAGAGTCTG
158	At4g32730	myb family transcription factor	CTCAGCGTCTTAATTACTTCAG	CCTTGGAGACTCTCTAGTGGAG
159	At4g37180	myb family transcription factor	CGAGGATCAAAGACAATCATTCC	GGTTTTGCCCTTTCAACATC
160	At4g14410	basic helix-loop-helix (bHLH) family protein	CCTTCTCTCGACGATGATTTCG	CCAATACTAAAGACTTCATCTGG
161	At4g28790	basic helix-loop-helix (bHLH) family protein	CCATGCTGGATGATGTTATAG	CCTAGGGAAAGGTATGAATGC
163	At4g13100	zinc finger (C3HC4-type RING finger) family protein	CCTCCTCACTAACTCGGAGAC	CCTCTCCTCCCCCTCGCAGC
165	At4g16420	transcriptional adaptor (ADA2b)	GCAGAACATGAAAGAAGAGTAC	GCTCAGCATCATTGTCATATTC
166	At4g25990	expressed protein	GTTCGGAGAAAGTGGTATGAGAG	CGTAGACCACTTAAATTCCTTG
167	At4g18020	pseudo-response regulator 2 (APRR2) (TOC2)	CCGATCATTGCATCACTACTAC	CCTTATGAACAACATGCTGC

168	At5g18240	myb family transcription factor	CCCATAAGTGATGCCCTTCAAATG	CCCAGCTGCACCTAGGTTCTG
169	At5g24520	transparent testa glabra 1 protein (TTG1)	GGTCTTCTTCGCAGCCTGATTG	GGAGCGCAAACCAAACCTAC
170	At5g28770	bZIP transcription factor family protein	GGTGTTTCCGTCTCCTCTCC	GGAAGAAGCAAGAGAGGCTTG
171	At5g18620	DNA-dependent ATPase, putative	CCATCAAAACGACCTTCGGG	CCTTGTCTCTAAGGAGCTTC
172	At5g60580	zinc finger (C3HC4-type RING finger) family protein	GGTACAGGTGCCATCGCTATATC	GGGCGAAGAGAACGACCAACGC
174	At5g13220	expressed protein	CCCATCGCAAGGAGAAAGTC	CCAAATCCAAAAACGAACATGG
176	At5g06960	bZIP family transcription factor (OBF5)	CCACGTCTTTGCAGTTGTAG	CCATCTGTTGAGACTGATGTTC
177	At5g59780	myb family transcription factor (MYB59)	GGACCGTGGACAGAACAGGAGG	GGCGTGAAGCTCAAGGACTAAAC
178	At5g67580	myb family transcription factor	GCGAATCACATTTGATCTATG	GCTTAAGAACTCCAGCTTTAAG
179	At5g48150	phytochrome A signal transduction 1 (PAT1)	CCTTGTCTCCGACAACTTTC	CCTAAGCTTCTCAACAGAGTTAG
181	At5g05550	expressed protein	GGAGAAGCAGAGAATGGAAG	GGATCCTCCAATTTCAATGAG
182	At5g16820	heat shock transcription factor 3 (HSTF3)	GGAGAATAATGACTTGGTATTGG	GGTAAATCTTTTATGTTTCTTC
183	At5g22000	zinc finger (C3HC4-type RING finger) family protein	CCTGAAACAAACAATGAGCAC	CCTTCTTCCTATGCTCCACAC
184	At5g23090	TATA-binding protein-associated phosphoprotein Dr1 protein, putative (DR1)	GGAGAGAGAAAATCCCTCCG	GGAAGAGAAGCGTCTTCCTTGG
185	At5g23090	TATA-binding protein-associated phosphoprotein Dr1 protein, putative (DR1)	GGATCCAATGGATATAGTCG	GGAGCAATCGTCCGTTTATCC
186	At5g12840	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	GGATTGATGGGAGCATATGG	GGCACGTGCTTTTCTTCGCC
187	At5g02470	DP-2 transcription factor, putative (DPA)	CAGTTTGTTTGTTTGTTTATAG	CCAATTTCAGAATCATCATC
188	At5g02840	myb family transcription factor	GCTGTTGATGAGAAACCTCAC	GCTGGTAGAGAGTTGTTGCG
189	At5g43270	squamosa promoter-binding protein-like 2 (SPL2)	CCTCTGGGATCCATAAGTTTTG	CCATCAATTTCCCACTCCATTG
190	At5g56370	F-box family protein	GGGCAAAATGTCAAATTAGG	GGAAGCAATGACAGTATGCGC
191	At5g18830	squamosa promoter-binding protein-like 7 (SPL7)	GGAGCCAGACAGTCTTGTTCAC	GGATCAGTCTCTTTTCCGCC
192	At2g46370	auxin-responsive GH3 family protein	CCTTTTTTCTCTCTCTCTCTATCC	CCACAGTTTTGTAAGTAAATGGC
193	At1g07350	transformer serine/arginine-rich ribonucleoprotein	CCTTGTCCTGGACCCATGGAC	CTCGAGCAGTTCTCAGCCCC
194	At3g49430	pre-mRNA splicing factor, putative	CCTCCGAGTATTGTTGGCTTCAGACC	CCTAATGTCACCGGGCAAGTTACC
195	At3g01150	polypyrimidine tract-binding protein, putative	GGCCAAAGAAGCACTGGAAGG	GGGTTCTGCCAACCTGCTGGTGG
196	At3g01150	polypyrimidine tract-binding protein, putative	CCATGAGATTGTTAACAATCAGAGTCC	CCAGCAGCTTTCTCAAATGTGGC
198	At4g36690	U2 snRNP auxiliary factor large subunit	GGATGAGCTTAGAGATGATGAGG	GGCCTGCCACTGGCTGACCATTGG
199	At1g79880	La domain-containing protein	GGGAGATGGAGAGAGAGTTGTGG	GGCTCCTTTCGGGCCTTCTTGG
200	At1g79880	La domain-containing protein	GGCCTCCTCCTTCAACGAAG	GGCCATCTTTGCTCTTGGTG
202	At3g13570	SC35-like splicing factor	GGAGGCCATTTGAGCAGTTTGG	GGTTTTCTTCAGCAAATACGACAG
203	At1g11650	RNA-binding protein 45 (RBP45)	CCAGAGCTCTGCTGCAGGGGTAAC	CGCAAACAACAAGCAGAGTGAC
204	At3g53500	zinc knuckle (CCHC-type) family protein	GGAAACATCCGCCTCTATGTTGG	GCGTCATCAGCATCACGAGG
205	At3g61860	arginine/serine-rich splicing factor RSP31	GGCAATTTCGAGTATGAAACTCGCCAGTCGG	GGAAAATTGTCGAGTTTGCGAATAG
206	At2g21660	glycine-rich RNA-binding protein (GRP7)	GGCGTCCGGTGATGTTGAGTATCGG	GGCTTTCTCATCCTTGAAGG
207	At2g47580	small nuclear ribonucleoprotein U1A	GGCGTTTAAGACCTTTAAGCACAAAGG	GGTTTTTGGACAAAGAATATTATTTGG
208	At1g09230	RNA recognition motif (RRM)-containing protein	CCGTCGGTTGTACTGTATATCAAAAACC	CTGCATTAGCCTCACACCAAGAC
209	At2g16940	RNA recognition motif (RRM)-containing protein	GGAGTCAGGAAAGAGAAAAGCTTGG	GTTCTCTGATCTCTTTCAGG
210	At4g02430	pre-mRNA splicing factor, putative / SR1 protein	CACATGCGTAAAGGAGGAGAAG	CCGAACATATTCATGAGAAAAC
212	At4g02430	pre-mRNA splicing factor, putative / SR1 protein	CCTGCAAAATCTACATCGAGATCTCC	CTGTCCTAGCTCGATGGGTCC
213	At5g53180	polypyrimidine tract-binding protein	GGATGTTGTGGGGAATGTTCTTCTGG	GGTATCCGGCTGTCTTCTCGAAAG
214	At1g31600	2OG-Fe(II) oxygenase family protein	CTCGCTTCGACCTCTCTTTC	CTAACCACAGAGACTTTGTTCC
215	At1g31600	2OG-Fe(II) oxygenase family protein	GACCATGTCCTGACCTTAAAGG	GGTAACAAGAAAAGCCCTGG
216	At2g42890	RNA recognition motif (RRM)-containing protein	GTACACTTACAAGATGCTGGTGG	GGAAGGGAACGATGTGCAATGG
217	At1g16610	SR45	CCTAAACCTGTCTCAGCTGCACC	CCTTCTAGGTGATAAGCCTCTCC
218	At2g30260	small nuclear ribonucleoprotein U2B	CCACCGAATCAATCAATCTACATCC	CAGTGACTTCACTAAAAGTAACCC

219	At4g25500	arginine/serine-rich splicing factor RSP40 (RSP40)	GGTGACCTGGAACGACTATTCAGG	GCGGTCAAGTGCTCGGATGG
220	At3g55460	SC35-like splicing factor, 30 kD (SCL30)	CCCTTTGAGAGGTTTGGACC	CCTGTTCATGCTTCTTTGAGCC
222	At5g06160	SF3a60/SAP61 Subunit	GGAGCCACCGTCCAGCAAGG	GTCGCAGTCTGGCCACCAAGCGCTG
223	At2g29210	splicing factor PWI domain-containing protein	GCAAATGAAATAATGAAGAAGAGGG	GTCAGCCCTTCTCCCATCCTCTGG
224	At1g15200	atPinin	CAAAAGAAATTGGAATTGCTTTTCC	CCTCACTTGTATCTTCTTCC
225	At3g53570	protein kinase (AFC1) (AME2)	CCGGCGATCAAATTACGACAGCGAC	CCGATGAGGGAATTCAACATTCC
226	At4g24740	protein kinase (AFC2)	GGTGTGAAGAAATACCGTGAGG	GGAGACTAACAGAATATTTTCTGG
227	At4g24740	protein kinase (AFC2)	CCTCATACTCACATGGATCGTCGTCC	CCATTTCCTTCCTCTCCCTATCCC
228	At4g32660	protein kinase (AFC3) (AME3)	GGGGTGCTGAGAAATATTTCAGG	GCTGTGAGACGCTCAGATGG
229	At4g35785	transformer serine/arginine-rich ribonucleoprotein	GGTGCATTAAATATCTCAACCAG	GGAGCTTTTCAAGCCAAGATAGTGG
232	At1g48960	universal stress protein (USP) family protein	CCACGGCGGCGCGTCTCCTCCGCC	CCAACGAGAAGCATCGAAGCACC
233	At5g54430	universal stress protein (USP) family protein	GGTGGAGATGATGATGGAGATG	GGTGATATATTACTTAGATCCCACG
234	At1g55870	CAF1 family ribonuclease	CTACATCAATGGATTTTCTTGCC	CCAACTTTAACTCTTCAGTTTCACC
235	At1g69080	universal stress protein (USP) family protein	GGAGAAGACGAATCTTGTG	GGCATGTTGTTTCTTCTGG
236	At1g04950	TATA box-binding protein-associated factor (TAF) family protein	GGATCTTCGTCGCTCAACG	GGAGCAAGCATAAGAGCAGCTTCAGG
237	At1g07830	ribosomal protein L29 family protein	CCATTCTCTGTGGCCGTCACCGTCGCC	CCGAATCGTCGAGGCAGCTGCTGC
239	At1g31500	endonuclease/exonuclease/phosphatase family protein	CTACAACTCTGCATCATCTTCC	CCTTCAACAGATTCAAATTTGC
241	At1g02090	COP9 signalosome complex subunit 7ii	CAAAAAACATCGTAAGGAAGC	CCCATCTTCTTCGTAGTCC
242	At1g60850	RNA pol subunit	CTAAATTGCTCAATTCTCTCGGCC	CCGGCAGGGACGTCAGGGAGATC
243	At2g33830	Auxin regulated protein (Dormancy)	CCGGACCTAAACCGGAGCATGGCC	CCGATCCTGGCGTCGTCGGAGTTCC
244	At5g09230	SIR2-family Protein	CATACAGTTCTGGTTTCAAACC	CCTGGTTGTGCTGCAGTGAACC
245	At5g46110	P/trioseP Translocator precursor	GGCGATCAACGGAGGAGAGAAAAG	GAAGAATCCAGTGACTAGCCATGG
246	At5g59440	Thymidylate kinase like	GAAACGGATCTGCTCGGTTTCAAG	GGTTCTGACACCAGAAGAAAAG
249	At1g72560	tRNA export mediator exportin-t	GGCTTCAACAAAACGGGAGTCACG	GGATGGTGAAAGCGAACCTTG
251	At1g37150	Holocarboxylase synthetase 2 (HCS2)	GGACAAAGAGTGATTGCTGAAG	CCATCAGGATGAAGCTCATAC
254	At5g50240	L-isospartyl methyltransferase	CCGCTACAATTCATCATCATC	CCTCTATCTAAAGCCTCCATAAC
256	At1g54080	UBP1A	CCAGAGGTTACTGATGCAGC	CCATCAGAGCTATGTTTGTCC
259	At3g17090	Phosphatase-2c	GGGACTTTTGTTGGTGTTTACG	GGTATTTCTTGCCACAGCTCTG
260	At3g26740	light regulated protein CCR-Like Circadian regulated	CCAACTATTCTTCTTCCTCTTCC	CCACTGATTACTTCGCATGC
261	At4g10100	molybdenum cofactor synthesis family protein (Auxin regulated)	CTCATGTGTGTGGTATTCACC	CAGTGTTAGATCAGGCACACC
262	At5g20040	IPP transferase 9 (IPT9)	CCGGAGTCTCTAAGAATGAGC	CCACGCATGAAGCTTCACTC
264	At5g65430	GF 14 Kappa isoform	CCGATCAGGCTTGGTTTGGC	CCGTCTTTTCTTCCATTAGACC
267	At2g42245	RNA binding protein-related	CAGGGTACATATGGGGACTCC	CCCTAATTAATCTTCTTACGC
270	At1g53650	RNA-binding protein	CCATGTTCAAGAAACTCAATCC	CCTTAGAAGCTCTTCCAGGTAACC
272	At3g23900	RNA recognition motif (RRM)-containing protein	GGATTATTCTTAAGTCATAGG	GGAGTGAAGGTGGTTCTTGG
273	At3g07810	putative / hnRNP	GCTAGAAGTTCATCTCCAGG	GGAAAAGAAGATAATCTAAAAG
274	At5g19030	RNA recognition motif (RRM)-containing protein	GGTTTTCGGATTCAGTGAGTGAAGG	GGCTTCGACAGCCGACTGTG
275	At5g19030	RNA recognition motif (RRM)-containing protein	GGACTCGGCAGTCACTAGGATACGG	GGAAATCGGAATGTGGTCTCCGG
277	At3g23830	glycine-rich RNA-binding protein, putative	CCTCTTTTCTCACTCACTGTTAC	CCATTTGAGCTCTGAGAAACCCC
278	At2g02570	atSPF30	GGCAGCTTTTGTCAGAGGATCCTAGG	GGAGTTGCTTCAGCACTAACTCCGG
279	At3g25840	protein kinase family protein	GGAGAGATTTTAGAAGAAGATGGG	GTTAGACTGCCTGCTGTTTGACGG
280	At5g11200	UAP56a	GGTACGTGGGAATACACAGTTCTGG	GGAGATGGTTCAATCTGTTGTAG
281	At5g47210	nuclear RNA-binding protein	CCAAAGTGTTTGAGTCCATGCAAC	CCACCTCTCCCGTTGTACCTC
282	At5g63120	ethylene-responsive DEAD box RNA helicase	GGTGACAAGACCCAATCGG	GGTTCGACCAATCCTATGG
283	At3g01540	DEAD box RNA helicase (DRH1)	CCAAGTACCACCACCTCTAATGTCC	CCTACCTTGCATAGCAATGGGCC
284	At4g33060	peptidyl-prolyl cis-trans isomerase	GGAGAAGAAGCTGAAGAAGAGG	GAAAAGCTTTTATCTTTTTGAG
285	At3g19840	WW domain-containing protein	CCATATTCTGGTTCTCATCC	CCAGGCATCTAACCGATTTCC

286	At1g67210	zinc knuckle (CCHC-type) family protein	GAACTCGATCCTCCTCCATGG	GAGGGCTAGCTTTTTCCAAG
288	At3g12570	expressed protein	CCATGTGTTGTACTAGTAGCC	CCATGGATAGCAGTGTTGAC
289	At3g12570	expressed protein	GGGATCATTTTGATAGGAGCG	GGTGATAGTTCTCCATAGACAGG
291	At2g46270	G-box binding factor 3 (GBF3)	CTCATTTCATTCTGTTTCTCC	CCTTAATCTTGACCTTCTAGC
292	At4g36730	G-box binding factor 1 (GBF1)	GATGAAAGTGTCACAGCGGG	GGCTTCATGGGCACCGAACCTTGG
293	At2g20180	basic helix-loop-helix (bHLH) family protein	CCGATGATGATTATGTCAACAACC	CCATAGAAGGAAGAAGCTTC
295	At2g02390	glutathione S-transferase zeta 1	GGCTTGATTATGAGTATATACCAG	GGTAACAAAGGTGGCTCAGGG
296	At1g67980	S-adenosyl-L-methionine:trans-caffeoyl-Coenzyme A	CACAAGATTAATTTTATCCATTCC	CCTTCACCAATTTTAGAAGCC
297	At3g44300	nitrilase 2	GGTGGATGTTTCGTATTGTCGG	GGTCAAGTGTAAAACATCTGG
298	At5g09880	RNA recognition motif (RRM)-containing protein	GGGATGTGCGATTGATCATGG	GGGCTTAACCATCACAGG
299	At5g11330	monooxygenase family protein	CCTCAGGCTCGGCAGATCATC	CCGAATCGAAGACAAACACCC
300	At5g59300	Ubiquitin-conjugating enzyme 7	CCGAAAATTCACAAAATCAGAACC	CAGGAGGTCCGATAATGGTAAC
301	At5g09790	PHD finger family protein / SET domain-containing protein	CCCATTGTTTGCTTGTCCCC	CCGTTAATGCAGAAGCAAGTGTTCC
303	At3g46460	ubiquitin-conjugating enzyme 13 (UBC13)	GGACTCAGTGGAGAAGAAGAAG	GAGGAACGCAACATTCTCCGG
304	At5g18800	NADH-ubiquinone oxidoreductase 19 kDa	CCGATCATCAATGGCCGCCGTGCC	CCACTGAGAATGATTTCCAAC
305	At1g01060	LHY late elongated hypocotyl - Myb-like DNA binding	CCCGGTGAGATGATAAGTC	CCATCTTTGATCTCCCCAAAC
306	At2g46830	CCA1 Myb-like DNA binding	GGACTGAGGAAGAACATAATAG	GGTTTACGCTTAGGCCGTGG
307	At3g11540	Spindly (Giberellin signal transduction protein)	GAGAAGGCTGCACTTGAGAGG	GAATCTGGATCTATCTTAAGG
308	At5g10140	FLC	GGAGGAACACCTTGAGACTG	GAGTCACCGGAAGATTGTCGG
309	At5g65060	MAF3	CAAGGAGTTACTAGAAATAGTCC	CCCGTGACATTCCTCTGTCACC
312	At5g13790	AGL15	GGGTTACTTAAGAAAGCTCG	GTAGGATGGAACATAGTGGG
313	At5g37055	SEF Zn finger HIT type	GGAAGAGATGTCGAACCGTCG	GCTGCTTTCAAGTAAGTCGG
314	At2g43410	FPA	GGGCTGGCTCTTACGATAACAG	GATGGCCTCCTCCAGTTTGG
315	At4g31120	SKB1 Methyltransferase	CTCAACGAAAGTCAACAACC	CCAACCCAGTACGAACGTCCC
316	At2g28550	AP2 transcription factor like protein;COLD	CGAAATTGGTAACTCCGGTTCC	CCAAGAGTAAAGTTGATATC
317	At2g28550	AP2 transcription factor like protein;COLD	CTTGGGAATCTCTTTATCGACC	CCCCAGTTACTCATCATCCC
318	At5g48560	putative bHLH transcription factor (bHLH078);COLD	GGACGCTCTTGTGTCAAAGG	GTTGAGATTCATTGTTGGGG
319	At5g65640	putative bHLH transcription factor (bHLH093);COLD	CCTCTGGTCAGAAACTCACC	CATTGTTCAATCTCCAAGCC
320	At5g24590	NAC2-like protein/turnip crinkle virus-interacting protein;COLD	CCCTGAGGTTACAACCACC	CCACATCCTTTCGTCATAGTTTCC
321	At4g27410	no apical meristem (NAM) family protein (RD26);COLD	CGATCTCTACAAGTTCGATCC	CTTGTAAATTCGACACAACACCC
322	At2g33480	putative NAM (no apical meristem)-like protein;COLD	CCGATGTTTGTAAATCCGATCC	CTGTCTCTTTCTCATTCTCC
323	At1g76580	Squamosa promoter binding protein-like 16 (SPL16);COLD	GGGTAACTACGCGTCAATGG	GATTCAACTCTTTGCAAATCGTGG
324	At5g43270	squamosa promoter binding protein-like 2 (emb|CAB56576.1);COLD	GGGATCCATAAGTTTTGAG	GCTTGAAGAATACAGAGAGG
325	At2g47890	putative CONSTANS-like B-box zinc finger protein;COLD	CCACGGATGTTCCTCCTCTGCC	CGAACCTTGCGTGATTCATACC
326	At1g69250	nuclear transport factor 2 (NTF2) family protein / RNA recognition motif (RRM)-containing protein;COLD	GGTCCTATCAAAGAAAACGGG	GGTCTGCAAGCATGAAGGGCG
327	At5g59950	RNA and export factor binding protein, putative;COLD	CTGCTCCATACCAATCAGCC	CCACTTCTATCAAAATGAAC
328	At5g52310	Dessication-responsive protein (RD29A) (COR78);COLD	GGTCAATGAGAAGGATCAAG	GATTGTGGTGACTCTTCAACGG
329	At4g17615	calcineurin B-like protein 1 (CBL1);COLD	GCAGTTCGGTTGTTGATGATGG	GGGGTGGAAAACATTGAGTG
330	At3g10300	calcium-binding EF hand family protein;COLD	CTCCTTATGTATCTATTCACC	CCGCTTCTGTCCTTATCAAACC
331	At3g10300	calcium-binding EF hand family protein;COLD	CTTCACTTTTCTTTAGTCTTC	GGTAAAACAGTGAGCATGAAG
332	At3g16800	protein phosphatase 2C, putative / PP2C, putative;COLD	GAATATTCATATCAGAGAAG	GGCTATCACATGTCCCCATGG
333	At3g16800	protein phosphatase 2C, putative / PP2C, putative;COLD	CCCGGTTCAGCTCTCGGTAGAC	CCTCTTACTATCTCCACTGCC
334	At1g01140	SOS2-like protein kinase PKS6/CBL-interacting protein kinase 9 (CIPK9);COLD	CCATCATTGCATGTGGTAGAGC	CAGTATTCCAGACTACATCC
335	At5g66210	calcium-dependent protein kinase;COLD	CAAAGACGGGTACATAACGCC	CCTCTTGCATCTAGTGCACC
336	At5g28080	mitogen activated protein kinase - like;COLD	GTTGAAGTTGATCCTACTGG	GAGCCTCTCTAGCTCTTGAGG

337	At5g28080	mitogen activated protein kinase - like;COLD	GTCAAGATTGGAGATCTTGG	GAGTCTAAGGGACACTGTGG
338	At5g57630	CBL-interacting protein kinase 21 (CIPK21);COLD	CAGATGCTAGAAAACTTTTCC	CCATACATCTACTGCTGCTCC
339	At4g13020	serine/threonine-specific protein kinase MHK;COLD	GTAGATACTTGTTCTTCAAGG	GGATGATTTAGCTTACGAAG
340	At5g63990	3'(2'),5'-bisphosphate nucleotidase;COLD	CGAGTTTCTTCGAGTCATACC	CTGCGTCGTAACGACGATTCC
341	At2g15970	similar to cold acclimation protein WCOR413 [Triticum aestivum];COLD	CACTATCGCTGCTAAGAACC	CTACGAAAGCAATCCATTTACC
342	At3g01090	putative SNF1-related protein kinase (KIN10);Dark, sugar, hypoxia	CCGAATTTTCTCCTCCGCC	CCAATACCAAGAGTTCTCCC
343	At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia	CCTGACTCAGCTCTGCGTCACC	CCCAATTCCAAGAGTTTTACC
344	At3g29160	SNF1-like protein kinase (AKin11);Dark, sugar, hypoxia	CCTGTTATTGGATAACCGGTTCC	CCAAGAGCCCATTTTCGATCAAC
345	At1g49730	protein kinase family protein;COLD	CTAAGCTGCATCACCGTAACC	CTTGATTTAATATCTCTATGAC
346	At4g23260	protein kinase family protein;COLD	GGGAAGTGTATCCCTTCTCTGG	GGAAAAGTTGCTTGTCGCCG
347	At4g34460	GTP binding protein beta subunit; ABA	CCAACTCCAGGTCTATCAGCC	CCTGTACACAAGGCACTTCC
348	At4g34460	GTP binding protein beta subunit; ABA	CGCCTCCAGCTCCTCGATACC	CTTGAGATGCACTGACAATCC
349	At5g40280	farnesyltransferase beta subunit (ERA1);ABA	CTTTAGGAGGTGACAAAGCCC	CCCTGGGTGAGTTCATCATCC
350	At1g77740	putative phosphatidylinositol-4-phosphate-5-kinase; ABA	CAGCGGTGAGGCTAAGAAACC	CAGGATCCACCGTGAATAGCTCCC
351	At2g18960	plasma membrane proton ATPase (PMA); ABA	CACTAGCAGAGCTATCTTCC	CGCTGAGAAGTCAAATTCCC
352	At5g57110	Ca2+-transporting ATPase-like protein; ABA	CTTACCGGAATTTTCTCTCC	CCCAGACTCCACATCGCCTCC
353	At1g27770	calcium-transporting ATPase 1, plasma membrane-type / Ca(2+)-ATPase isoform 1 (ACA1) / plastid envelope ATPase 1 (PEA1); ABA	CGAAGCACTTCGAACTCTTTGCC	CTCACCAAAGTATGCTTATCC
354	At5g23450	diacylglycerol kinase family protein;sphingosine kinase (AtLCBK1); ABA	CTTTTGCTGATACACTTCTCTCC	CGTCTAAGCGACTGTTGTGCC
355	At3g16785	phospholipase D, putative; ABA	CTCTCTTTGTGGTCCGAACACC	CCCAAATCGATCGTTGTGTGTCCC
356	At3g56860	UBA2a; RNA binding protein; ABA	CACTAAATGTATCATGTATCC	CGTTGAGAAGCTTGCACCAC
357	At1g54100	aldehyde dehydrogenase, putative / antiquitin, putative; COLD; SALT; DESSICATION;ABA	CATTTCTTCGATACGATCAACC	CCATATTTTAGCTGCTTCCTC
358	At1g09000	NPK1-related protein kinase, putative (ANP1); OXIDATIVE; COLD	CCGTTACAGATAAATAACACC	CCATTACCAGGACTTTCGTC
359	At2g38170	calcium exchanger (CAX1); COLD; SALT	GGAAATGCAGCTGAACATGCTGG	GGACCAGTCCCTTCATGTAG
360	At3g06510	glycosyl hydrolase family 1 protein; COLD	CTCTACCAATGGGTGATTCC	CCCGATTCACTGTATTCATC
361	At4g13850	Glycine-rich RNA binding protein AtGRP2- Like; COLD; OSMOTIC	CCTTAAGGGATGCTTTTGCTC	CCTCCTCTGTTTAGAAACCACC
362	At4g26070	mitogen-activated protein kinase kinase (MAPKK) (MKK1) (MEK1);COLD; WOUNDING; BIOTIC	CCTTCAAACTTGCTAATCAATC	CCTGCGTCGGTGAAGTAAGCC
363	At5g24270	calcineurin B-like protein, putative / calcium sensor homolog (SOS3); SALT	GAGTTTGGTGAATTTGTCCGG	GGCAAAGTCATGTTCTTGATG
364	At5g67030	zeaxanthin epoxidase (ZEP) (ABA1); ABA; DESSICATION; HEAT	CCATCGATGCTTGACTGGGTCC	CCGAAACGCAACAATCGTCGCC
365	At4g34000	ABA-responsive elements-binding factor (ABF3); ABA	GGTGCAGTTCTGGAGAAAGTG	GCATCCCATTCCCCATGGCTGG
366	At5g25610	dehydration-responsive protein (RD22)	CCTCTGATCTGTCTTCTTGGTTC	CCAACGTTCACGGCGGTTCC
367	At1g71860	protein tyrosine phosphatase; COLD; SALT	CCACCTAGTCTCGGCCCAATC	CCCTCTCTAAGCAGACGAACC
368	At1g78290	protein kinase 1-like protein; SALT; OSMOTIC	CTTCCCAAGAAGATCAAATCC	CCTATGGTTCATGATTTCTC
369	At4g31720	transcription initiation factor IID (TFIID) 23-30kDa subunit (TAF2H) family protein; SALT	CGTCTCAGCTCAATTCTCTTCC	CTCTTAGCCAAGTAGTGCTCC
370	At5g35410	CBL-interacting protein kinase 24 (CIPK24) / serine/threonine protein kinase (SOS2); SALT	CAGCAGAGTTTTCTTGTCCACC	CCAAGGATCTTTCTTGATTCC
371	At5g37370	pre-mRNA splicing factor PRP38 family protein (SRL1); SALT	GGACGGATGACGACAATGGG	GGGCAGTGTCTTCTGAGCCACGGG
372	At1g76460	RNA recognition motif (RRM)-containing protein	GGTCGATCCAAAGGATATGG	GGAAGTGGTTGCTGATACGG
373	At3g13224	RNA recognition motif (RRM)-containing protein	GTTGGTGGTATACCCTCAACGG	GGGTGACTACCGTAAGAAGG
374	At4g36960	RNA recognition motif (RRM)-containing protein	GTTTAGGGCATCCTTGTGGG	GTAATCCTTAAGCCCATCGG
375	At3g20270	lipid-binding serum glycoprotein	CTCAGGGATTACTTACTACC	CTCCGCGGAATAAATTAGCCC

376	At1g64625	bHLH transcription factor	GGGAGAAGTAGCTTCATCCGG	GAGTAGATCCTAGCTGAACAACG
377	At3g51530	F-box family protein	CCGTCGTCGTATGTCTCTCTC	CCCATACTCCAACATAAACACC
378	At3g62190	DNAJ heat shock N-terminal domain-containing protein	CCTGATGATCAGAAGCTTGTTGCC	CCGAGGAACCCCAGTCTTGAC
379	At5g04275	MIR172b	GTTTGTTGTGTGTGAGACTTTGG	AGATGAGCTTTCTTCCATGG
380	At5g08185	npcRNA 78; MIR162a	GTCCATTTGGTTTCATAAGG	GCTTTCCCAGAAAAGTAATCGG
381	At5g53450	fibrillin fused protein kinase	GGGAAAGAATCAAACTTTTG	GGTTTCGTTTATAACCAATG
382	At1g44910	FF domain-containing protein / WW domain-containing protein; splicing factor-like	GCAAAATTCAAGATAGACTGG	GGCTGTAAGGATGCCTGCAG
383	At2g43640	signal recognition particle 14 kDa family protein / SRP14 family protein	CCTTTTTACCGAAGAGACTCC	CCCTTCTCTTTGCTTTTCTC
384	At4g02200	drought-induced-19-like 1	GTGTCACCATATCGATGAGG	GACATCTCTATGCTGAGTGG
385	At2g43810	putative small nuclear ribonucleoprotein polypeptide F	GGCTGCTTCATCGAGCGGAAG	GTTGAGCTTGACAACAACTGG
386	At4g01250	WRKY family transcription factor;COLD	CCGGTAGCGTCACTAGCAAACCC	CTTGTTTACGGGCTAAACAACC
393	At2g26150	HsfA2	GGAAATGGAGGAAGAAACGG	GCCTCAACCTAACTACCTCAG
411	At3g26744	ICE1;COLD	GGAGAAATAGTGACGGTGAG	GATCTTGTGGATGTGGTTGG
412	At4g29810	MAP kinase kinase 2;COLD	GGATTCAGCAATAATCTCAAG	GCTCAGCTGATCATCAGCTGG
413	At2g33120	Putative Synaptobrevin (SAR1) NUP160;COLD	CCTTCTTCTAACAACAAGTTTACC	CCATATCTCTTGTTGAAATCC